EFFECT OF THE COMPOSITION OF THE GASEOUS AND AQUEOUS ENVIRONMENTS ON THE SURVIVAL OF TUBERCLE BACILLI IN VITRO

BY RENÉ J. DUBOS, Ph.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

(Received for publication, September 25, 1952)

One of the mechanisms which has been invoked to account for the progressive disappearance of tubercle bacilli from certain types of tuberculous lesions (closed cavities, cold abscesses, etc.) is the low oxygen tension prevailing in these areas. As far as is known, tubercle bacilli depend upon molecular oxygen for growth, and the rate and extent of their multiplication decrease rapidly in proportion as the oxygen tension falls below that of ordinary air (1-4). It appears, moreover, that anaerobic conditions are unfavorable to the maintenance of their viability, and metabolic activity (5, 6).

Low oxygen tension is, of course, only one of the characteristics of the closed tuberculous lesion which renders its physicochemical environment different from that prevailing in the rest of the body. Among other differences which can be surmised to be of importance for the multiplication and survival of tubercle bacilli, are the concentration of CO₂ in the aqueous and the gaseous phases and the presence of organic acids produced by anaerobic metabolism and of other unusual substances released from the tissues by autolytic processes.

The experiments to be described in the present paper were designed to test the effect on the viability of tubercle bacilli of some of the factors presumed to be present and to operate in the tuberculous lesion.

Experimental Methods

1. Cultures.—Five strains of tubercle bacilli of human and bovine origin were used in the present study (MV, H37Rv, R1Rv, BCG-P, BCG-T). Their origin, virulence, and other characteristics have been fully described in other papers from this laboratory (7).

2. Bacteriological Techniques.—The special media and methods used for the cultivation of the bacilli under aerobic and anaerobic conditions are described in detail for each experiment.

The numbers of viable organisms were determined by plating appropriate dilutions of the bacterial suspensions on oleic acid–albumin agar (8). Dilutions were made in tenfold steps in 0.1 per cent bovine albumin (bovine plasma fraction V obtained from Armour Laboratories, Chicago). The oleic acid–albumin agar was prepared according to methods given in reference 8; the plates were kept at 37.5°C. for 24 hours before use not only to test for sterility, but also to remove excess moisture from the agar surface. Drops of culture dilutions were de-
Viability of Tubercle Bacilli

VIABILITY OF TUBERCLE BACILLI

358

posited on the agar surface from capillary Pasteur pipettes delivering approximately 0.025 ml. The plates were incubated at 37.5°C. for 2 weeks in plastic bags to minimize evaporation.

For each test mixture, at least two and sometimes four samples were used. Two independent sets of dilutions were carried out for each sample, and two platings were made for each dilution. The variability in colony counts from one determination to the other never exceeded fivefold. The numbers given in the tables are averages of these counts expressed to the nearest whole exponential value of 10.

RESULTS

Viability of Tubercle Bacilli in the Gaseous Environment Created by Oat Seedlings and Microbial Cells Respiring Anaerobically.—A crude, but convenient technique often used by bacteriologists to create an anaerobic environment consists in adding germinating oats to the vessel from which oxygen is to be removed. In practice, the removal of oxygen under these conditions is brought about, not only by the oat seedlings, but also by the microbial population which becomes established in the oats mash. Together, oats seedlings and contaminating microorganisms utilize the available oxygen so completely that anaerobic fermentation becomes evident in the container within 24 to 48 hours after it has been hermetically sealed. The effect of the gaseous environment thus created on the viability of tubercle bacilli was determined as follows:

Experiment 1.—

Two strains of tubercle bacilli were used: the attenuated strains R1Rv and BCG-P. They were grown for 10 days in tween-albumin medium to which had been added 0.2 per cent glucose (the concentration of tween 80 in the medium was 0.05 per cent; that of serum albumin, 0.5 per cent.) The cultures were distributed in 2 ml. fractions into small test tubes (10 mm. inner diameter).

The cotton plugs in some of the tubes were impregnated with melted paraffin in order to prevent evaporation. The contents of these tubes served as "aerobic" controls, since the amount of oxygen present in the gaseous phase of the culture under these experimental conditions was sufficient to allow abundant growth of the tubercle bacilli.

Other tubes were placed in an environment rendered anaerobic by the following technique. To a number of large tubes (30 mm. diameter), there was added approximately 7.5 gm. of oats and 15.0 ml. of water. A small tube containing 2 ml. of culture was introduced into each of these large "oats tubes." Then the large tubes were closed tightly with a screw cap into which had been inserted a thick rubber washer.

The "aerobic tubes" and "oats tubes" were kept at room temperature for 48 hours in order to allow time for the establishment of anaerobic conditions in the latter; then all of them were transferred to the incubator at 37.5°C. At intervals of time, two tubes of each set were removed and the numbers of living bacilli which they contained were determined by the technique described under Experimental Methods. The results are presented in Table I.

Whereas the numbers of viable bacilli in the tubes kept under aerobic conditions remained approximately constant throughout the period of observation, the cultures in the tubes maintained in the gaseous environment created by the germinating oats were sterilized within 3 weeks. In fact, only a small
fraction of the tubercle bacilli originally present in the anaerobic tubes were able to develop into colonies when transferred to agar medium after but 1 week of anaerobic incubation.

At the end of the experiment, drops of the contents of each tube were spread on glass slides and the films were stained by a modification of the Ziehl-Neelsen technique (9). Microscopic examination of the stained films revealed striking differences between the aerobic and anaerobic cultures. The former still consisted of well formed acid-fast bacilli, whereas the latter showed evidence of extensive autolysis of the bacterial cells. Only a small percentage of acid-fast rods could be seen amidst non-acid-fast ghost cells against a background of amorphous non-acid-fast debris. Similar experiments were carried out with six other strains of tubercle bacilli, with essentially the same results. Furthermore, the viability of the cultures was tested by transfer not only on agar media, but also into liquid media under aerobic conditions. In no case was it possible to obtain growth from cultures maintained for 4 weeks or longer in an atmosphere rendered anaerobic by germinating oats. Even more striking was the fact that these cultures failed to establish an infection in mice, even when highly virulent strains were used. Thus 0.2 ml. of culture MV and of H37Rv which had been kept in the “oats tubes” for 4 and 5 weeks were injected by the intravenous route into groups of three mice. None of the animals died, and when they were sacrificed 4 weeks later no living tubercle bacilli could be recovered from their spleens or lungs. These results acquire special significance in view of the fact that amounts as small as $10^{-4}$ ml. of aerobic cultures of these strains in the same tween-albumin medium are always capable of establishing an infection in mice (7).

While the atmosphere in the tubes containing the germinating oats was certainly anaerobic, it does not follow necessarily that absence of oxygen had been the cause of the death of the tubercle bacilli in these tubes. For, the oats and accompanying microorganisms had produced large amounts of CO$_2$ and other volatile substances as a result of their metabolism, thus modifying the gaseous environment in ways other than by mere removal of oxygen. The following experiment was designed to test whether the high CO$_2$ pressure in the “oats tubes” had been responsible for the death of the bacilli in the preceding experiment.

**Experiment 2.**—

The procedures used in Experiment 1 were modified as follows: To some of the tubes containing both culture and germinating oats, there were added inner tubes containing strips of paper immersed in a 20 per cent solution of NaOH to absorb the CO$_2$ from the anaerobic atmosphere. As control, the alkali was added under the same conditions to other sealed large tubes from which the oats had been omitted. The numbers of viable tubercle bacilli were determined at weekly intervals (Table I).
The results presented in Table I show that addition of concentrated alkali to the aerobic tubes did not affect the viability of the bacilli under the conditions of the experiment. However, addition of the alkali to the system which had been rendered anaerobic by the germinating oats and accompanying bacteria decreased appreciably the rate at which the bacilli died in this gaseous environment. It is probable that this protective effect was due to the removal of CO₂ (and perhaps of some other volatile acids produced under anaerobic conditions of metabolism). It was observed that the contents of the "oats tubes" without NaOH were under positive gas pressure whereas this was not the case when concentrated alkali had been added to the system.

**The Viability of Tubercle Bacilli in a Gaseous Environment Free of Oxygen and CO₂**—The addition of alkali to solutions of pyrogallic acid has long been used by bacteriologists as a procedure for establishing an anaerobic environment. Since oxidation of the pyrogallic acid takes place only at alkaline reactions, this technique necessarily brings about removal of CO₂ at the same time as that of oxygen. The use of sodium pyrogallate made it possible therefore to follow the viability of tubercle bacilli in a gaseous environment free of these two gases.

### Table I

**Effect of Gaseous Environment on the Viability of Tubercle Bacilli**

<table>
<thead>
<tr>
<th>Agents used to modify the gaseous environment</th>
<th>Strain of T.b.</th>
<th>No. of viable tubercle bacilli present after following periods of time, in wks., at 37.5°C.</th>
</tr>
</thead>
</table>
| Germinating oats (anaerobic)                 | BCG-P         | 10⁸  
| " " and concentrated NaOH                    |               | 10⁸ 10⁷ 10⁴ 10⁴ 0 0  |
| Pyrogallic acid and concentrated NaOH (anaerobic) |             | 10⁸ 10⁷ 10⁴ 10⁴ 10⁴ 10⁴ 10⁴  |
| Concentrated NaOH (aerobic)                  |               | 10⁸ 10⁷ 10⁴ 10⁴ 10⁴ 10⁴ 10⁴ 10⁴  |
| Controls (aerobic)                           |               | 10⁸ 10⁷ 10⁴ 10⁴ 10⁴ 10⁴ 10⁴ 10⁴ 10⁴  |
| Germinating oats (anaerobic)                 | R1Rv          | 10⁸ 10⁷ 10⁴ 0 0  |
| " " and concentrated NaOH                    |               | 10⁸ 10⁷ 10⁴ 10⁴ 10⁴ 10⁴ 10⁴ 10⁴  |
| Pyrogallic acid and concentrated NaOH (anaerobic) |             | 10⁸ 10⁷ 10⁴ 10⁴ 10⁴ 10⁴ 10⁴ 10⁴  |
| Concentrated NaOH (aerobic)                  |               | 10⁸ 10⁷ 10⁴ 10⁴ 10⁴ 10⁴ 10⁴ 10⁴ 10⁴  |
| Controls (aerobic)                           |               | 10⁸ 10⁷ 10⁴ 10⁴ 10⁴ 10⁴ 10⁴ 10⁴ 10⁴ 10⁴  |

---

- not done.

* The figures give the numbers of colonies (calculated to the nearest whole exponential value of 10) recovered per milliliter of culture.
Experiment 3.—

The experimental procedure was similar to that used in Experiment 1 except for the fact that 3 gm. of pyrogallic acid and 10 ml. of 20 per cent NaOH instead of oats were added to the large tubes. As controls, some of the tubes received only the strips of paper impregnated with NaOH solution. The numbers of living bacilli were determined at weekly intervals (Table I).

As indicated by the results presented in Table I the bacilli placed in an atmosphere which had been rendered anaerobic by sodium pyrogallate and had also been deprived of CO₂ died at approximately the same rate as those placed in the vessels containing germinating oats and NaOH. It appears, therefore, that lack of oxygen alone can be the cause of rapid death of tubercle bacilli, and that the lethal effect of the anaerobic environment is accelerated still further in the presence of CO₂.

Effect of Na Lactate and Alanine on the Viability of Tubercle Bacilli Placed under Anaerobic Conditions.—In order to investigate the effect of various physiological substances on the viability of tubercle bacilli maintained under conditions of anaerobiosis, the cultures were cultivated in a simplified medium and the substances to be tested were then added to the cultures grown aerobically in this medium before beginning anaerobic incubation. The following experiment deals with the effect of sodium lactate on viability.

Experiment 4.—

The culture of tubercle bacilli used in this experiment (strain BCG-P) was grown for 10 days at 37.5°C in a medium similar to that used in Experiment 1 except that ferric citrate, hydrolysate of casein, glucose, and glycerine had been omitted from it. The fully grown culture was distributed in 1.8 ml. amounts into small test tubes and to some of these was added 0.2 ml. of either 5, 2.5, 1.25, or 0.62 per cent solution of sodium lactate (DL) giving final concentrations of 0.5, 0.25, 0.125, or 0.062 per cent of lactate. Control tubes received physiological saline instead of sodium lactate. Two tubes of each mixture were placed in a small vacuum desiccator (1 liter capacity) to which was added 30 gm. of pyrogallic acid and 30 ml. of 4 per cent NaOH. Three such desiccators were prepared. They were opened after 1, 3, or 4 weeks' incubation at 37°C and the number of bacilli in each of the tubes was determined. In addition, some of the tubes, having received either saline or sodium lactate, were kept aerobically in tubes with screw cap tops, but without reducing agents, and were tested at the same time as the anaerobic sets (Table II).

The results presented in Table II make clear that the addition of sodium lactate to the aqueous environment accelerated the death of bacilli placed under conditions of reduced oxygen tension, whereas the bacilli kept under aerobic conditions survived during the whole period of observation even in presence of the highest concentrations of lactate tested.

Comparative Viability of Various Strains of Tubercle Bacilli Placed in Different Media under Anaerobic Conditions.—It has been repeatedly observed in our laboratory that stock cultures of attenuated strains of tubercle bacilli
appear to lose their viability more rapidly than do the virulent cultures. For this reason an experiment was instituted to test whether differences could be recognized among various strains with regard to the length of their survival under anaerobic conditions. As it had been found in the preceding experiment that the addition of sodium lactate to the medium accelerated the rate of death of BCG (Table II), the new tests were designed to determine the influence of this substance as well as that of another metabolite—namely alanine,—on the viability of several strains of tubercle bacilli.

Experiment 5.—

Five strains of tubercle bacilli were compared: MV, H37Rv, R18v, BCG-P, and BCG-T. Their pathogenicity and other characteristics have been described in earlier publications (7).

### TABLE II

<table>
<thead>
<tr>
<th>Sodium lactate added</th>
<th>Conditions of incubation</th>
<th>No. of viable bacilli present after following intervals of time, in wks., at 37.5°C.</th>
</tr>
</thead>
<tbody>
<tr>
<td>per cent</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>0.4</td>
<td>Anaerobic</td>
<td>10**</td>
</tr>
<tr>
<td>0.2</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>0.1</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>0.05</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>0</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>0.4</td>
<td>Aerobic</td>
<td>&quot;</td>
</tr>
<tr>
<td>0</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
</tbody>
</table>

*The figures give the numbers of colonies (calculated to the nearest whole exponential value of 10) recovered per milliliter of culture.

They were grown for 10 days in the simplified medium (without glucose, glycerine, ferric citrate, or hydrolysate of casein) used in Experiment 4. The cultures were distributed in 1.8 ml. amounts into small test tubes to each of which was added 0.2 ml. of 2 per cent solution of Na lactate or DL-alanine or saline. Some of the tubes were kept under aerobic conditions, the others were placed in desiccators with sodium pyrogallate as described in Experiment 4. The numbers of viable bacilli in the different mixtures were determined at weekly intervals (Table III).

In confirmation of the results presented in Table II, those in Table III reveal that tubercle bacilli can survive for several weeks under anaerobic conditions provided the medium is somewhat deficient in substances that they can metabolize. In contrast, the bacilli die rapidly in the anaerobic environment if metabolites such as alanine, and particularly sodium lactate, are added to the medium. It must be noted, however, that these substances do not in any way decrease viability under aerobic conditions. Although the response
of tubercle bacilli to the composition of the gaseous and aqueous environments was qualitatively the same for the five strains tested, there were quantitative differences among them. In Table III the strains are arranged in order of decreasing virulence for mice and it appears that this order also corresponds to the order of decreasing resistance to anaerobiosis in the presence of lactate and alanine. However, further experiments with more strains, and more quantitative bacteriological techniques, will be required to determine whether there exists a correlation between the virulence of the cultures and the length of their survival under anaerobic conditions.

**DISCUSSION**

When cultures of tubercle bacilli grown in a medium of complex composition are subsequently placed in an anaerobic environment at 37.5°C., the numbers of viable cells decrease rapidly. Within a few weeks, the bacilli are found to be unable to multiply *in vitro* and *in vivo* as demonstrated by the failure to obtain subcultures or even infection when they are transferred aerobically into new

---

**TABLE III**

**Effect of Na Lactate (0.2 Per Cent) and Alanine (0.2 Per Cent) on the Viability of Various Strains of Tubercle Bacilli under Anaerobic Conditions**

<table>
<thead>
<tr>
<th>Culture</th>
<th>Substance added</th>
<th>No. of viable tubercle bacilli present after the following intervals of time, in wks., at 37.5°C.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>MV</td>
<td></td>
<td>No. of viable tubercle bacilli present after the following intervals of time, in wks., at 37.5°C.</td>
<td>Aerobic</td>
<td>Anaerobic</td>
<td>Aerobic</td>
<td>Anaerobic</td>
</tr>
<tr>
<td>Mv</td>
<td>Lactate</td>
<td>10^9</td>
<td>10^9</td>
<td>10^8</td>
<td>10^8</td>
<td>10^8</td>
</tr>
<tr>
<td></td>
<td>Alanine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>10^8</td>
</tr>
<tr>
<td>H37Rv</td>
<td>Lactate</td>
<td>10^9</td>
<td>10^9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Alanine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>10^9</td>
</tr>
<tr>
<td>R1Rv</td>
<td>Lactate</td>
<td>10^9</td>
<td>10^9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Alanine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>10^9</td>
</tr>
<tr>
<td>BCGP</td>
<td>Lactate</td>
<td>10^9</td>
<td>10^9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Alanine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>10^9</td>
</tr>
<tr>
<td>BCG-T</td>
<td>Lactate</td>
<td>10^9</td>
<td>10^9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Alanine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>10^9</td>
</tr>
</tbody>
</table>

* The figures give the numbers of colonies (calculated to the nearest whole exponential value of 10) recovered per milliliter of culture.
culture media—liquid or solid—or injected into mice by the intravenous route. Moreover, prolonged incubation in the proper anaerobic environment causes the bacilli to undergo partial lysis. Within a few weeks the bacillary suspension is found to consist largely of non-acid-fast ghost cells and of amorphous debris. Death of the bacilli and autolytic changes appear particularly rapid when the CO₂ tension of the gaseous environment is high and when certain metabolites—sodium lactate and alanine for example—are added to the aqueous phase.

The toxic effect exerted by sodium lactate on tubercle bacilli under anaerobic conditions throws some doubt on the validity of the commonly held view that these organisms do not possess the metabolic equipment for glycolysis (5, 10, 11). It is true that organic acids never accumulate in any significant amounts when the bacilli are incubated with glucose aerobically or anaerobically. The findings reported in the present paper make it likely, however, that the lactate that they could produce anaerobically would become toxic to them under these very conditions and thus might prevent further glycolysis. On the other hand, it is also known that the bacilli utilize lactate in the presence of oxygen (5, 12, 13) and probably destroy it as fast as they produce it under aerobic conditions. New techniques are therefore needed before it is possible to determine whether tubercle bacilli are actually deficient in glycolytic mechanism.

The finding that tubercle bacilli lose their viability in the presence of lactic acid under anaerobic conditions may throw some light on certain aspects of the pathogenesis of tuberculosis. There exists in and around tuberculous lesions a state of oxygen deficiency which probably causes inflammatory cells (and perhaps also fixed tissue cells) to produce in these areas large amounts of organic acids (14-19). The scanty information available suggests that the concentration of lactic acid may in some cases be high enough to interfere with the multiplication of tubercle bacilli even if sufficient free oxygen for this is available in the environment (20). The results of the present study indicate further that, under conditions of oxygen deficiency, the presence of sodium lactate may result not only in inhibition of growth, but also in a bactericidal effect. In fact, it seems worth considering whether a similar concatenation of circumstances may not occur within monocytic cells which have phagocytized tubercle bacilli, since it has long been known that following phagocytosis the intracellular pH can become very acidic (21-23).

Finally, the deleterious effect of high CO₂ tension on tubercle bacilli under conditions of anaerobiosis is worth mentioning. Immobilization of the whole or part of a lung naturally results in interference with oxygenation, and also in an accumulation of CO₂ in the immediate vicinity of the immobilized part. It is known that gases in stagnant tissue contain approximately 6.5 per cent CO₂, a concentration much in excess of the optimum for the growth of tubercle bacilli in the presence of oxygen and probably sufficient to hasten their death under anaerobic conditions (24, 25). It seems probable, therefore, that gaseous
conditions are extremely unfavorable to the survival of tubercle bacilli in the collapsed lung, and this could account in large part for the therapeutic effects of artificial pneumothorax.

Thus, evidence is accumulating that the mechanisms of defense of the body against tuberculous infection operate, not only through the ordinary processes of immunological reactions (humoral or cellular), but also by creating at the focus of infection a physicochemical environment which interferes with the multiplication and survival of tubercle bacilli (20, 26). The peculiarities of the inflammatory process as modified by allergy, by hormonal control, and by anatomical accidents, such as communication with a bronchus or blood vessel, determine the physicochemical characteristics of each particular tuberculous lesion, and hence the nature of the environmental factors which affect the multiplication and survival of the bacilli. In this sense, it is possible to speak of a local immunity different from the general immunological state of the body, and yet influenced by it. This may account for the commonly observed fact that the bacilli can thrive and produce progressive disease in one part of an organ, while being held in check in another area of the body, even indeed in the same organ.

**SUMMARY**

Tubercle bacilli die much more rapidly under anaerobic than under aerobic conditions. Anaerobically, the rate of death is accelerated by increased CO₂ pressure, and when certain metabolites (lactate for example) are present in the medium.

Death of the bacilli under certain conditions of anaerobiosis is accompanied by loss of ability to take the acid-fast stain and by progressive cellular disintegration.

These findings are discussed in relation to (a) the glycolytic equipment of tubercle bacilli, (b) their fate in tuberculous lesions.

**BIBLIOGRAPHY**

366 VIABILITY OF TUBERCLE BACILLI