THE GROWTH OF BACILLUS MEGATHERIUM IN RELATION TO THE OXIDATION-REDUCTION POTENTIAL AND THE OXYGEN CONTENT OF THE MEDIUM

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It is now believed by many bacteriologists that the growth of anaerobic bacteria is controlled by the oxidation-reduction potential of the medium. This seems to have partially supplanted the old belief in the rôle of oxygen. In spite of the attractiveness of the new view, there seems little experimental basis for its adoption, aside from certain observations like those in relation to the ability of anaerobes to grow under atmospheric pressure in the presence of reducing substances. But in ordinary culture media the oxygen content and the oxidation-reduction potential are so intimately related, that observations like the one just mentioned may be misleading. The problem is yet to be investigated. The effect of oxygen and the effect of the potential must be segregated and studied separately.

The question may also be asked: what about aerobic bacteria? Is their growth controlled by the oxidation-reduction potential, or by the oxygen content of the medium? Do, for instance, anaerobic and aerobic spore formers constitute two fundamentally different classes of bacteria, or do they form a single series, the members of which differ from one another by a maximum and a minimum oxidation-reduction potential, or by a maximum and a minimum oxygen content of the medium?

The present investigation deals with the question in one of the aerobic spore-forming bacteria, *Bacillus megatherium.*

EXPERIMENTAL PROCEDURE

Our line of attack was first to familiarize ourselves with the oxidation-reduction peculiarities of certain common media under atmospheric, as well as under reduced, air pressure, and to study the conditions under which *Bacillus megatherium* grows in these media. The media we used most frequently were meat infusion broth and ordinary nutrient broth, but we have often used various synthetic media and peptone solutions.

Then we sought to influence the potential of our media by adding various oxidizing or reducing substances, so that their potential could be raised or lowered as independently from their oxygen content as possible. We sought reducing substances which, when added to a culture tube, would reduce its potential, in the air, below a certain figure found to correspond to inhibition of growth under vacuum, and oxidizing substances which would give the medium, under a known inhibiting vacuum, a potential superior to that certain figure which, in the normal medium, corresponds to inhibition of growth. These oxidizing and reducing substances must be of low toxicity so that they may be added in concentrations sufficient to produce the desired degree of shift in potential.

TECHNIQUE AND APPARATUS

Our potential measurements were made in the usual way with a potentiometer with respect to the saturated calomel electrode. We used a platinum electrode made of fine wire (gauge 30). A short piece of wire was sealed by both ends to a glass tube, thus forming a loop. Connection between the culture tube and the calomel electrode was made by means of a saturated potassium-chloride-agar bridge dipping into a tube of saturated, aqueous potassium chloride into which dips also the side tube of the calomel half-cell. The complete cell can be represented as follows:

Hg/HgCl / Saturated KCl / Saturated KCl agar / medium /Platinum

The medium under investigation was placed in a test tube of 21 to 22 mm. internal diameter, so that a 10- cc. portion formed a column of 31 to 33 mm. In this way we reduced considerably the

errors which may result from the position of the electrode in the medium, due to differences in oxygen content between liquid layers at different levels.

In order to measure the potential under reduced pressure, the above cell was mounted on a square, shellacked wooden test tube block and placed inside of a pyrex vacuum desiccator, and connection with the potentiometer was made by means of copper wires through a rubber stopper. The outfit was arranged so that two medium half-cells could be switched alternately into circuit with the calomel half-cell. In this way all our experiments were run in duplicate.

In addition to the wire leads, the rubber stopper carried also a thermometer and a small, closed-arm manometer which enabled us to read the temperature and pressure inside of the desiccator irrespective of the atmospheric pressure. The manometer had a side arm, closed with a glass stopcock, through which evacuation was carried.

The test tube block carried also one or more tubes of distilled water for the purpose of maintaining a known vapor pressure inside of the desiccator, and two tubes of the medium under investigation inoculated from a week old slant culture of *Bacillus megatherium* to test for growth. Whenever the medium investigated was not ordinary meat infusion, two more tubes of similarly inoculated normal meat infusion were also added for comparison.

I. The oxidation-reduction potential of sterile, normal meat infusion broth

Although one finds in the literature data on the oxidation-reduction potentials of culture media, we found it desirable to secure first-hand information about the oxidation-reduction properties of the media we used, both in the air and under vacuum.

In this, as in other cases, the drift in potential readings was recorded, but we considered the true potential of the medium a certain constant value obtained after a variable length of time. Our zero time refers merely to the first reading taken immediately after the outfit was assembled.

In the air the potential of the sterile meat infusion broth is

nearly equal to that of the saturated calomel electrode, usually a few millivolts more negative. *Under reduced air pressure*, the potential drops considerably with the pressure. Figure 1 contains the results of measurements made in the air and under relatively high vacuum.

Growth of Bacillus megatherium in normal meat infusion broth. *In the air, Bacillus megatherium* grows profusely in normal meat

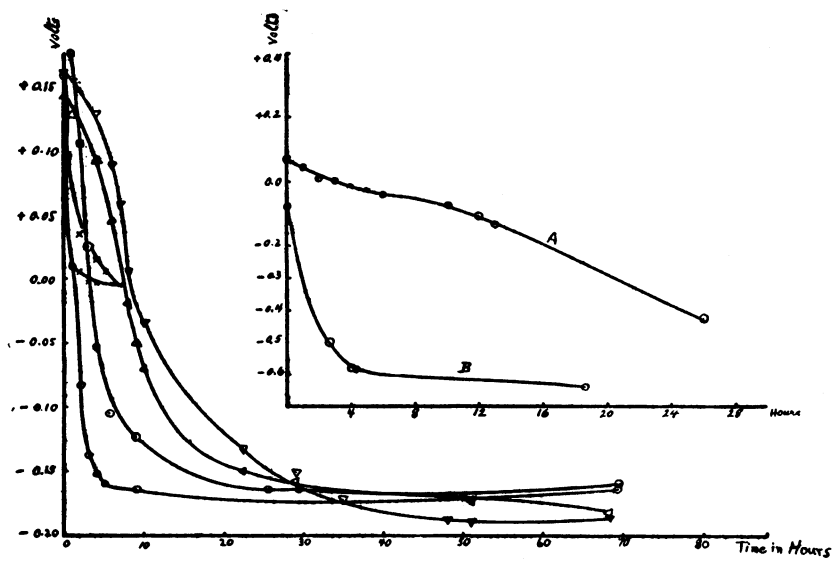

FIG. 1. Time–potential diagrams of sterile meat infusion broth under atmospheric and reduced pressure, and of meat infusion broth inoculated with *Bacillus megatherium*. Readings are with respect to the saturated calomel electrode. × = atmospheric pressure; ⊙ = 20 mm. of air pressure; ▽ = 15 mm. of air pressure.
Insert: *A* = inoculated with a loopful of cell suspension. Incubated under an air pressure of 330 mm. Hg. at 26°C. *B* = heavily inoculated. Incubated under atmospheric pressure at 30°C.

infusion broth of the proper reaction, pH 5.6 being the lower pH limit. If the inoculum is slight, no change in the potential of the medium is observed until growth begins. A heavy inoculum shifts the potential immediately to the negative side. As growth goes on, the potential becomes more and more negative. The diagrams inserted in figure 1 give potential readings on cultures of *B. megatherium* under atmospheric pressure and under moderate vacuum.

If the inoculated tubes be incubated in the desiccator and the air pressure within the desiccator reduced, it will be found that good growth still occurs at a considerable vacuum, and not until the air pressure inside of the desiccator has dropped below 10 mm. of mercury is growth inhibited. At this point, the potential of the medium drops to −0.160 volt or below. Even at that low pressure, microscopic examination often reveals a few young cells, although the tube may look perfectly clear. Determination of absolute limits of growth, whether it be in the anaerobic jar or in the presence of poisons, is a very difficult task, and it is our intention to study this question further.

II. The meat infusion-sulfite medium

In our search for a reducing substance which would reduce the potential of meat infusion below −0.160 volt under atmospheric pressure, we tried several compounds like cystein, glucose, and ferrous sulfate. None of these was satisfactory and they were dropped when, on trying sodium sulfite, we found the interesting results given below.

After having satisfied ourselves of the efficiency of sodium sulfite in reducing the potential in the air, we studied the inhibiting action and toxicity of the compound for *Bacillus megatherium*. We prepared a series of infusion sulfite media containing from 0.1 to 1.5 per cent of sodium sulfite. After sterilization of the infusion broth, the sulfite was added, and the tubes allowed to stand in the laboratory for twenty-four hours before inoculation. The idea was to allow the medium to absorb oxygen. The inoculated tubes were incubated at 30°C. and examined for growth after four days. Our results consistently showed growth in 0.25 per cent and no growth in 0.27 per cent of sodium sulfite. The higher concentrations showed growth on prolonged incubation up to 0.45 per cent, but not in 0.5 per cent or above.

These results suggest that inhibition by 0.27 to 0.45 per cent sodium sulfite is only temporary because of its reducing action, the inhibiting effect being removed on oxidation of the sulfite. Only in concentrations of 0.5 per cent and above does the sulfite become toxic, destroying the inoculum.

Potential measurements on 0.27 per cent sulfite infusion are given in figure 2, and it is seen that the potential is below −0.160 volt, the same reading obtained in normal infusion broth under inhibiting vacuum.

Was this a coincidence? or was it of fundamental significance? A clue to the mechanism was suggested to us by the observation that the potential of the 0.27 per cent sulfite infusion was the same in the air as under high vacuum. It was then that we began

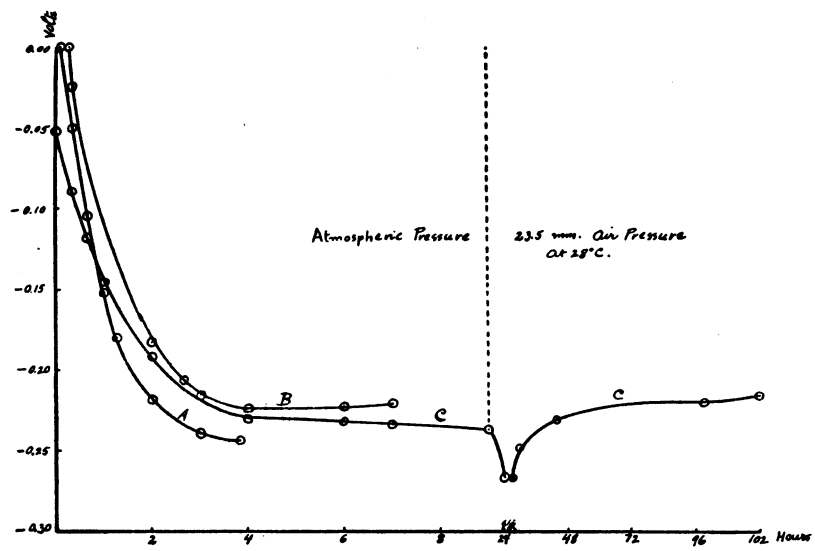

FIG. 2. Time—potential diagrams of meat infusion broth of pH 6.8, containing 0.27 per cent sodium sulfite. Readings refer to the saturated calomel electrode.

to investigate the oxygen content of our culture media as will be indicated below.

III. The ferric ammonium citrate infusion broth

In our search for an oxidizing substance which would keep the potential of the culture medium high, even under high vacuum, we spent considerable time investigating compounds like sodium nitrite, sodium nitrate, various ferric salts, 1-naphthol, 2-sulfonate indophenol, kindly furnished by Professor W. M. Clark, and litmus. Various concentrations of these compounds were tried,

but all were unsatisfactory, either because of inefficiency or because of toxicity. We also tried various synthetic media which gave us higher potentials than biological media. But the most satisfactory results were obtained from media, synthetic or biological, to which ferric ammonium citrate was added.

Ferric ammonium citrate can be added to culture media in relatively high concentrations without hindering growth. But this citrate is very sensitive to light and may give a low or a high potential, depending on whether it has or has not been exposed to

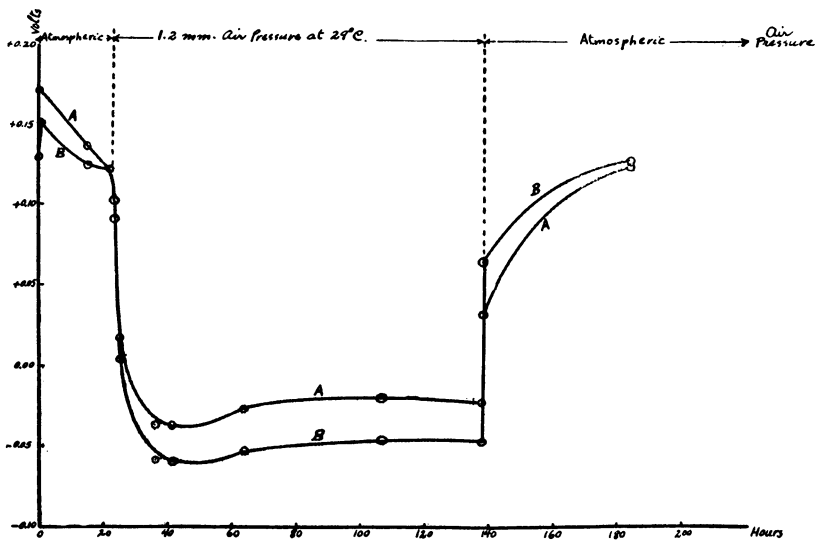

FIG. 3. Time—potential diagrams of ferric ammonium citrate nutrient broth of pH 7, protected from light during preparation and measurement. Readings refer to the saturated calomel electrode.

light. We were, therefore, very careful in preparing the medium in relative darkness and in wrapping all containers in black paper throughout the experiments. The ferric ammonium citrate media have a high potential in the air and remain considerably above −0.160 volt in the desiccator. Although we ran many experiments with synthetic media containing the citrate, most of our experiments were with ordinary meat extract broth containing 0.5 per cent of ferric ammonium citrate.

Figure 3 contains the results of a few experiments with ferric ammonium citrate media.

TABLE 1

Oxygen content of various culture media

Cubic centimeter of oxygen in 20 cc. of medium at 30°C.

MEDIUM	DUPLICATE DETERMINATIONS	AVERAGE
Meat infusion broth; pH 7	0.07 0.07	0.07
Nutrient broth; pH 7	0.08 0.11	0.095
Nutrient broth + 0.5 per cent ferric ammonium citrate; pH 7	0.12 0.12	0.12
Dox's solution +0.1 per cent ferric ammonium citrate; pH 6	0.19 0.16	0.175
Meat infusion +0.27 per cent sodium sulfite; pH 7	0.00 0.00	0.00
Nutrient broth made 0.05 molar ferrous potassium citrate	0.00 0.00	0.00

TABLE 2

Summary of results

MEDIUM	AIR PRESSURE	POTENTIAL	OXYGEN CONTENT IN 20 CC. MEDIUM	GROWTH
		volts	*cc.*	
Meat infusion broth	Atmospheric	−0.003 to −0.005	0.7	+++
Meat infusion broth	10 mm. Hg	<-0.160		−
Meat infusion broth +0.27 per cent sodium sulfite	Atmospheric	<-0.200	0.00	−
Meat infusion broth +0.27 per cent sodium sulfite	23.5 mm. Hg	<-0.200		−
Nutrient broth +0.5 per cent ferric ammonium citrate	Atmospheric	About +0.100	0.12	++
Nutrient broth +0.5 per cent ferric ammonium citrate	< 10 mm. Hg	>-0.050		−

IV. The oxygen content of various media

The results of the investigations outlined above are summarized in table 2. These results may be stated as follows:

Bacillus megatherium grows profusely in meat infusion broth of the proper reaction under atmospheric pressure. The potential of sterile, neutral meat infusion broth in the air is nearly 0.000 referred to the saturated calomel half-cell. Growth can be inhibited at about 10 mm. of mercury at 30°C., and the potential of neutral meat infusion broth at that pressure is −0.160 volt or below.

When sodium sulfite is added to meat infusion broth, the potential drops considerably, even in the air. The lowest concentration of sulfite that inhibits growth in four days is 0.27 per cent, and the potential of neutral meat infusion broth containing 0.27 per cent of sodium sulfite is about −0.250 volt in the air and does not change on evacuation.

When ferric ammonium citrate is added to ordinary nutrient broth and the medium protected from light, the potential is over +0.100 volt in the air and growth is profuse. On evacuation to a pressure of about 10 mm. of mercury, this potential drops to about −0.040 volt and growth is inhibited.

If the potential of the medium were the determining factor, it would not be possible to reconcile the results obtained. In normal meat infusion broth excellent growth can be obtained when the medium is given a potential of −0.040 or much below. Why is it then that no growth takes place in the citrate medium at that potential, when we know that this medium is very suitable for growth under atmospheric pressure? The fundamental difference between the two media is that a very high vacuum is required to bring the citrate medium down to −0.040 volt, while normal meat infusion reaches that potential at a relatively low vacuum. The action of oxygen is here very strongly suggested. But we have, on the other hand the 0.27 per cent sulfite medium which does not allow growth in the air, and its potential is comparable to that which is found in normal meat infusion under inhibiting vacuum.

This apparent contradiction made it indispensable to study the the oxygen content of sulfite and other media.

The apparatus used to determine oxygen was Van Slyke's blood gas apparatus, with alkaline pyrogallate to absorb the oxygen. We modified the technique to suit our purpose and used

20 cc. samples. All media were analyzed for oxygen after standing in the 30° incubator for twenty-four hours, and all oxygen determinations were made at that temperature.

The results are recorded in table 1, and they show that the sulfite medium which inhibited growth of *Bacillus megatherium* was free from oxygen or, more conservatively, its oxygen content in a 20-cc. sample was below the sensitivity of the apparatus which reads to 0.01 cc. This explains why the potential of 0.27 per cent sulfite broth is the same in the air and under vacuum.

The other figures show also an interesting comparison between the oxygen content of the other media tested. Dox's solution containing ferric ammonium citrate, which was used in some unreported experiments, contains more oxygen than nutrient citrate broth, and the latter contains more than normal nutrient broth. Normal meat infusion has the lowest oxygen content of all. Biological media are never in equilibrium with the atmosphere, they are constantly absorbing oxygen, and their oxygen consumption is proportional to their reduction potentials.

SUMMARY AND CONCLUSION

The present investigation shows that normal meat infusion broth of pH 7 has a potential nearly equal to that of the saturated calomel electrode. In a vacuum that inhibits the growth of *Bacillus megatherium* ($\leq$ 10 mm. of air pressure), this potential drops to -0.160 volt or below.

If sodium sulfite is added to meat infusion broth, growth is inhibited when the concentration of sulfite reaches 0.27 per cent. The potential of this medium is nearly -0.250 volt in the air and does not change in the vacuum.

When ferric ammonium citrate is added to nutrient broth in a concentration of 0.5 per cent, growth takes place readily. In the air, the potential of such a medium is over $+0.100$ volt, and about -0.040 volt under a vacuum that inhibits the growth of *Bacillus megatherium*. Even at that relatively high potential of -0.040 volt, the organism does not grow.

The oxygen contents of the above media, after standing for twenty-four hours at 30°C., were in cubic centimeter of oxygen in

20-cc. samples as follows: meat infusion broth of pH 7, 0.07 cc.; meat infusion broth +0.27 per cent sodium sulfite, 0.00 cc.; meat extract broth +0.5 per cent ferric ammonium citrate, 0.12 cc.

These results show that the limiting factor in the growth of *Bacillus megatherium* in vacuum is the oxygen content and not the oxidation-reduction potential of the culture medium.