CCXLVII. THE MECHANISM OF BACTERIAL ANAEROBIOSIS.

III. THE OXIDATION-REDUCTION POTENTIAL OF THE BUTYRIC BACILLUS IN PRESENCE OF SULPHYDRYL COMPOUNDS.

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It has been shown in a previous publication [1933] that anaerobic bacteria can be cultivated under aerobic conditions in symbiosis with aerobes or in presence of certain compounds which act as hydrogen acceptors. Among them sulphydryl compounds may be mentioned which were found to be active not only in the oxidised but also in the reduced form. This is no doubt due to the fact that during growth of the bacteria one of the forms is partially converted into the other one no matter which of them was added to the culture-medium. According to Hopkins [1931], some dynamic equilibrium seems to exist in the tissues between the oxidised and reduced forms of glutathione, and this may be regarded as a support to the behaviour of the cysteine-cystine system.

We are still quite ignorant with regard to the mechanism of action of sulphydryl compounds on the growth of anaerobic bacteria though several attempts have been made to throw some light upon the problem. Hosoya and Kishino [1925], and independently of them Quastel and Stephenson [1926] have shown that aerobic growth of strict anaerobes can proceed in the presence of sulphydryl compounds. According to these authors, the action of sulphydryl compounds consists not only in removal of free oxygen through oxidation of these compounds but also in the establishment of a certain level of the reduction potential which is indispensable for the growth of the anaerobic bacteria. This point of view is supported by Aubel *et al.* [1929] who have shown colorimetrically that growth of strict anaerobes can proceed only in a medium the redox potential of which varies from $r_H 0$ to $r_H 12$. Clark [1924] has also advanced the idea that a certain reducing potential of the medium is indispensable for the growth of anaerobic microbes, and Fildes [1929] and Knight and Fildes [1930] have shown colorimetrically that germination of the spores of *Bacillus tetani* becomes possible only at a certain level of the redox potential of the medium which should not exceed the value of $E_H = +0.01$ volt.

Plotz and Gelofo [1930] maintained the reduction potential of the medium at a definite level by introducing into it certain oxidants or reductants. Cultivation of various anaerobic bacteria in the above media has shown that growth of the culture can proceed only within the range of r_H lying between 0 and 14. The potential of the medium whatever be its initial value is reduced by growth of the bacteria to $r_H 5.5$ irrespectively of the species used.

Establishment of a definite level of the reduction potential has been sometimes regarded, however, not as a factor which is necessary for the growth of

anaerobic microbes but as a result of this growth. In this connection a paper by Pasteur [see Plotz and Geloso, 1930] should be mentioned in which the reducing capacity of the butyric bacillus was shown by decoloration of indigo-blue. Among more recent publications are those of Tarozzi [1905] and Potter [1911] on *Bacillus coli*, of Gillespie [1920] on soil bacteria, and finally that of Lepper and Martin [1930]. Some authors however do not regard establishment of a certain negative oxidation-reduction potential as a necessary condition of bacterial growth. Coulter and Isaaks [1929] by passing a finely divided current of oxygen into the culture-medium raised the reduction potential of the medium to a positive level which did not effect any inhibition of growth. Hewitt [1930] regards the bacteria themselves as responsible for the negative potential of the medium as the result of metabolic processes on the cell-surface.

In a new publication by Quastel and Wooldridge [1929] Quastel gives up his former concept of the negative potential as an indispensable factor of anaerobic growth. According to the new concept of Quastel, much more weight should be attached to the chemical composition of the medium and to the nature of catalysts operating on the surface of the bacterial cell, while the potential itself is regarded as a factor of secondary importance.

It will be seen from the above that the problem of bacterial anaerobiosis is yet an open one, though its theoretical as well as practical importance can hardly be doubted. Solution of this problem promises to give an insight into bacterial metabolism in general and into the mechanism of formation of bacterial products such as toxins and enzymes, or spore formation *etc.* It would also render it possible to change the bacterial metabolism at will and to study the possibility of cultivation of anaerobic microbes on synthetic media.

In the present publication an attempt has been made to find out the correlation between the part played by sulphhydryl compounds in the aerobic growth of anaerobic microbes and the oxidation-reduction potential of the system. The latter was determined electrometrically by means of the usual technique.

METHODS.

The butyric bacillus (a pure culture of *B. saccharobutylicus*, anaerobic strain) was cultivated in full test-tubes of 200 ml. capacity containing 100 ml. of culture-medium. The rubber-stopper had four holes through two of which the platinum electrode and KCl-agar bridge were introduced while the other two served for passing the stream of purified nitrogen. As culture medium a 2 % sugar bouillon was used, or a bouillon fermented by yeasts in which the content of peptone amounted to 2 % instead of the 1 % usually employed, or a 0.25 % sugar bouillon with the same content of peptone. Reduced glutathione, cysteine or cystine was added to the medium so that the final concentration of the substance amounted to 0.02 %. The above concentrations of glucose and of sulphhydryl compounds were low enough not to influence the redox potential of the medium. The p_H of the medium was kept at 7.0–7.1, and the temperature of the thermostat at 37°. Experiments were carried out mostly under anaerobic conditions while occasional aerobic experiments served to follow up the reduction potential in the presence of air. Anaerobiosis was insured by passing through the test-tube a stream of nitrogen freed from traces of oxygen by passing it through a heated quartz tube filled with metallic copper. The potential was followed up electrometrically every $\frac{1}{2}$ –1 hour in the course of 6–7 days, and occasionally up to 3 weeks.

RESULTS.

The results of the experiments are illustrated by the curves of Fig. 1.

It will be seen from the data obtained that both the pure medium and the medium containing the bacterial culture are represented by the same type of curve either under anaerobic or aerobic conditions. It will be noted also that the anaerobic potential is more negative than the aerobic, whereas in the presence of

Fig. 1. Variation of E_H with time. A, 2 % sugar medium; aerobic. B, 2 % sugar medium; anaerobic. C, 2 % sugar medium + bacteria; aerobic. D, 2 % sugar medium + bacteria; anaerobic.

bacteria it shifts still more to the negative side. Growth of bacteria in a sugar-containing medium is initiated within the first hour after inoculation. This is accompanied by an abrupt fall of the potential curve, while on the next day the potential of the pure medium, and two days later that of the culture, becomes more positive reaching the initial value or even exceeding it, though growth of the bacteria still continues. The character of the curves does not render it possible to discriminate between the behaviour of the medium itself and that of the culture, the difference, if any, being a quantitative one.

The 2 % sugar medium was replaced therefore by one containing less sugar, *viz.* one containing 0.25 % sugar in a bouillon which had been previously fermented with yeast and contained 2 % of peptone. The results obtained with this medium are illustrated by the curves of Fig. 2.

It will be noted that there is no drift to negative potential in the bouillon itself either containing sulphydryl compounds or not. The aerobic potential

however is more positive than the anaerobic. If however a culture of bacteria is introduced into the medium (and also in symbiosis with sarcina) the potential shifts to the negative in the course of bacterial growth, attaining a value which lies close to $E_H = -280$ mv. This level of potential remains constant for a considerable period of time as well as the p_H of the medium.

Fig. 2. Variation of E_H with time. A, 0.25 % sugar bouillon; aerobic. B, 0.25 % sugar bouillon; anaerobic. C, 0.25 % sugar bouillon + bacteria; anaerobic. D, 0.25 % sugar bouillon + cysteine; aerobic. E, 0.25 % sugar bouillon + cysteine; anaerobic. F, 0.25 % sugar bouillon + cysteine + bacteria; aerobic. G, 0.25 % sugar bouillon + cysteine + bacteria; anaerobic. H, 0.25 % sugar bouillon + glutathione; aerobic. I, 0.25 % sugar bouillon + glutathione; anaerobic. K, 0.25 % sugar bouillon + glutathione + bacteria; aerobic. L, 0.25 % sugar bouillon + glutathione + bacteria; anaerobic.

No drift of potential has been noted in the pure medium, which is due to the fact that it contains a rather low concentration of glucose and of sulphhydryl compounds. The oxidation-reduction curves obtained in bacterial cultures which contain cysteine or glutathione are nearly identical: the drop of the anaerobic cysteine curve precedes that of the glutathione curve, though the latter becomes stabilised at a more negative level than the former. The data of Table I illustrate the rate of fall of potential during bacterial growth.

A concentration of 0.25 % glucose in the medium suffices to induce anaerobic growth of the butyric bacillus, which sometimes may become possible even under aerobiosis when cultivating the organism in a deep layer of liquid. In order to study the part played by sulphhydryl compounds it was necessary therefore to cultivate this organism in a medium absolutely free from glucose.

The curves of Fig. 3 refer to the new culture medium (bouillon, the broth of which had been fermented with yeast). In this medium the butyric bacillus

Table I.

	E_H mv.	Time		E_H mv.	Time
Cysteine + bacteria	+ 189	9 a.m.	Glutathione + bacteria	+ 164	9 a.m.
	+ 97	11 a.m.		+ 67	11 a.m.
	+ 40	11.30 a.m.		+ 45	11.05 a.m.
	+ 12	12 noon		+ 9	11.25 a.m.
	- 1	12.05 p.m.		- 42	12 noon
	- 56	12.35 p.m.		- 62	12.30 p.m.
	- 96	1 p.m.		- 106	1 p.m.
	- 162	2 p.m.		- 169	2 p.m.
	- 199	3 p.m.		- 197	3 p.m.

Fig. 3. Variation of E_H with time. *A*, Fermented bouillon; anaerobic. *B*, Fermented bouillon + bacteria; anaerobic. *C*, Fermented bouillon + cysteine; anaerobic. *D*, Fermented bouillon + cystine; anaerobic. *E*, Fermented bouillon + cysteine + bacteria; anaerobic. *F*, Fermented bouillon + glutathione; anaerobic. *G*, Fermented bouillon + glutathione + bacteria; aerobic. *H*, Fermented bouillon + glutathione + bacteria; anaerobic.

does not grow even under anaerobiosis; this is shown by the curve running nearly parallel with that of the culture medium itself. However the addition of 0.02 % of sulphhydryl compound to the medium induces growth of bacteria both aerobically and anaerobically; this is shown by the course of the curves. This illustrates the part played by sulphhydryl compounds in the aerobic growth of anaerobic microbes. In presence of a negligible amount of a sulphhydryl compound the bacteria are enabled to react with the medium by decomposing the nutriment supplied by the medium and oxidising it, whilst the energy liberated by oxidation is used up for synthetic processes going on in the cells. The rôle of the sulphhydryl compounds seems therefore to consist in promoting the inter-

action between the bacteria and the medium. Little is known as yet about the nature of this activation but it will be noted that sulphydryl compounds are known to activate various enzymic processes, *e.g.* proteolysis and conversion of methylglyoxal into lactic acid and also to exert a certain influence upon the Pasteur-Meyerhof reaction through which some correlation between respiration and fermentation is maintained. It seems probable that we are dealing here with an activation of some processes which are necessary for establishment of oxidation-reduction equilibria.

Bamberger *et al.* [1933] have shown indeed that the irreversible system of pyruvate-lactate becomes reversible in presence of an enzyme and of a reversible system consisting of methylene blue-leucomethylene blue. Its E_H can be correctly determined, and it proved to fall into the same negative range of potential as that found in the above systems. A tentative suggestion may therefore be made that in the systems reported above a similar redox equilibrium might be established, the more so as pyruvic acid is known to appear in the fermentation processes as an intermediate product.

Cultivation of anaerobic microbes on synthetic media would be an important contribution to the study of the mechanism of anaerobiosis, and application of the redox potential method to the study of general bacterial metabolism seems to be very suggestive.

SUMMARY.

1. Sulphydryl compounds promote the growth of anaerobic bacteria either under aerobiosis or anaerobiosis.

2. Growth of the bacteria is accompanied by a drop of the redox potential, which acquires a definite value within two or three days in a medium containing cysteine or glutathione. The anaerobic potential corresponds to $E_H = -280$ mv. while the aerobic potential is more variable. In presence of glutathione it varies from -280 to -180 mv.; in presence of cysteine it amounts to -220 mv.

3. The drop of the anaerobic potential in presence of cysteine either precedes that of the glutathione medium or coincides with it, while under aerobiosis the former occurs first.

The courses of both the curves were found to be identical and this suggests that the mechanism of action is similar in both cases. In absence of bacteria and in a medium containing microbes which do not grow no drop of potential has been found.

4. Growth of bacteria is probably not due to establishment of a negative reduction potential. It is initiated prior to this when the potential is still positive and in a sugar-containing medium continues even at a positive potential level.

5. Growth of bacteria in the presence of sulphydryl compounds and of sugars should not be attributed to the reduction potential of the medium, since the negative potential does not become established in a medium containing small amounts of these substances in absence of bacteria. The drop of potential is due to some interaction which takes place between the bacteria and the medium in the course of metabolic processes going on in the bacterial cell.

6. The composition of the medium (sugar or sulphydryl compounds) influences the course of single phases of the bacterial metabolism, this being shown by the shape of the corresponding curves (see Figs. 1 and 2).

7. Sulphydryl compounds seem to exert some peculiar activating effect upon the interaction between the microbes and the medium as a result of which certain coupled reversible systems of the type lactate-pyruvate might appear.

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