CULTIVATION OF ANAEROBES AND OXIDATION-REDUCTION POTENTIALS[1]

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In the cultivation of anaerobes in fluid media exposed to air it has been customary for some time to poise the medium at a favourable oxidation-reduction potential level by the addition of reducing agents or to retard diffusion of oxygen by the addition of agar to the medium. Allwin and Baldwin (1930) reviewed the use of reducing agents some ten years ago. Since that time, other agents have been introduced, particularly ascorbic acid (Klinger and Guggenheim, 1935) sodium thioglycollate and sodium formaldehyde sulfoxylate (Brewer, 1940). Spray (1936) reviewed earlier work on the use of agar and successfully used it in the cultivation of clostridia.

Brewer (1940) combined the use of reducing agents and agar in a successful medium which has been widely used (McClung, 1940; Reed and Orr, 1941).

These additions produce at least theoretical complications. To broth or peptone solutions, active oxidation-reduction systems, there is added another oxidation-reduction system in the form of a reducing agent; the establishment of equilibrium with the atmosphere is delayed by the addition of agar; a further oxidation-reduction system is introduced with the inoculum. At the same time we have no precise information on the limiting oxidation-reduction potentials for the growth of any species, though Quastel and Stephenson (1926), and Fildes (1929) have probably approached these limits for two species as closely as the complexity of the system will permit.

In the present paper an attempt is made to evaluate the influence of the various ingredients of the media on the O/R potential as measured at a polished platinum electrode and to correlate the potential with the minimum inoculum, of various species of *Clostridium*, to induce growth.

1. O/R POTENTIAL DETERMINATIONS

Oxidation-reduction potential measurements were made in the conventional manner, essentially as described by Knight (1931) and by Thornton and Hastings (1929) except that a Beckman vacuum tube potentiometer was used.

Test tubes 6 x 1 inch, served as electrode vessels. Two electrodes, consisting of 3 cm. lengths of 26 gauge platinum wire coiled and sealed in glass tubes, were supported in the test tubes in 4-hole rubber stoppers. The two remaining holes provided for a KCl-agar bridge and a hooded tube through which the inoculum was introduced with a long Pasteur pipette. Twenty ml. amounts of media provided a depth of 6 cm. in the tubes. Electrodes were cleaned in hot chromate

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cleaning mixture, followed by liberal amounts of distilled water. They were autoclaved in the medium to be tested, or in distilled water and transferred to the media. In tests the electrode vessels were arranged on a shelf on the inside of the door of a 37°C. air incubator. KCl-agar bridges from as many as 20 vessels dipped into an oblong trough of saturated KCl. The standard half cell, a saturated calomel electrode, was immersed in this trough. Leads from the electrodes were carried through switches, on the outside of the incubator door, to the potentiometer.

E. M. F. readings were referred to the normal hydrogen electrodes in calculating Eh values. No corrections for pH were attempted. The sterile medium was always pH 7.5 to 7.6 and no significant changes occurred during the periods of observations. In growing cultures of clostridia the reaction gradually became acid to a maximum pH of approximately 6.5 in sugar-free media and 5.0 in media with 1 per cent fermentable sugar.

(a) *Sodium thioglycollate medium.* The greater part of this study has been devoted to a simple medium previously shown, Reed and Orr (1941), to support luxuriant growth of the gas gangrene-tetanus group of clostridia:

Bacto or Proteose peptone	20	grams
Na_2HPO_4	2	grams
Agar	1	gram
Sodium thioglycollate	1	gram
Methylene blue	0.002	gram
Water	1,000	ml.

Adjusted to pH 7.6 with NaOH

This medium gives a well-poised negative O/R potential, Eh −0.175 to −0.200 volt with different batches (fig. 1). Methylene blue in the medium is reduced to its colorless phase during autoclaving. On cooling the surface layer is oxidized to the blue form in a few hours and the blue layer gradually extends but the bottom half of a deep tube of medium remains colorless for several weeks. In contrast, the peptone medium without thioglycollate gives a strongly positive O/R potential, EH +0.225 to +0.250 volt (fig. 1). Methylene blue in the medium if reduced in the autoclave is oxidized to the blue form on cooling.

Smaller concentrations of sodium thioglycollate than 0.1 per cent in the peptone medium produce a less negative O/R potential and very much less efficient poising of the medium (fig. 2). When 1 ml. of air is rapidly bubbled through 20 ml. of peptone medium containing 0.1 per cent sodium thioglycollate there is a slight positive drift in potential followed by a rapid return to the preaeration level. But with a similar bubbling of 1 ml. of air through media with 0.01 per cent sodium thioglycollate there is a rapid rise followed by a more gradual positive movement until the Eh of the medium approximates that of the peptone solution without added reducing agent (fig. 2).

(b) *Other reducing agents.* Cysteine or ascorbic acid in similar percentage concentrations in this peptone medium produces approximately the same Eh levels as are produced by sodium thioglycollate. Sodium formaldehyde sulfoxylate

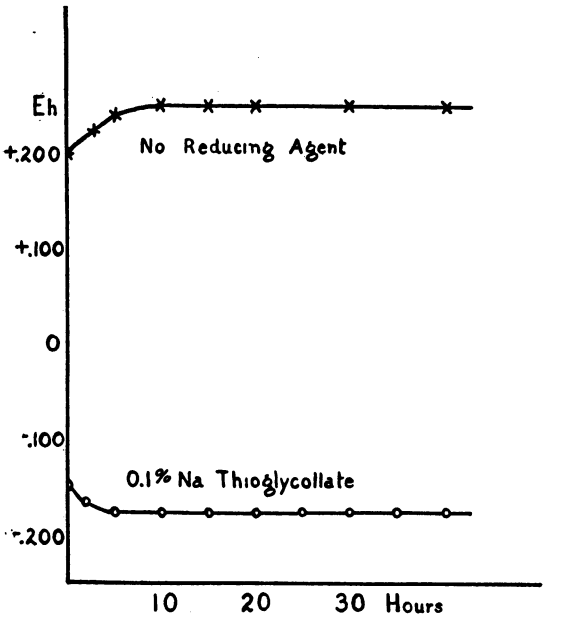

FIG. 1. GRAPH INDICATING THE O/R POTENTIALS OF STERILE PEPTONE BROTH WITH AND WITHOUT 0.1 PER CENT SODIUM THIOGLYCOLLATE
Ordinates represent Eh and abscissa time

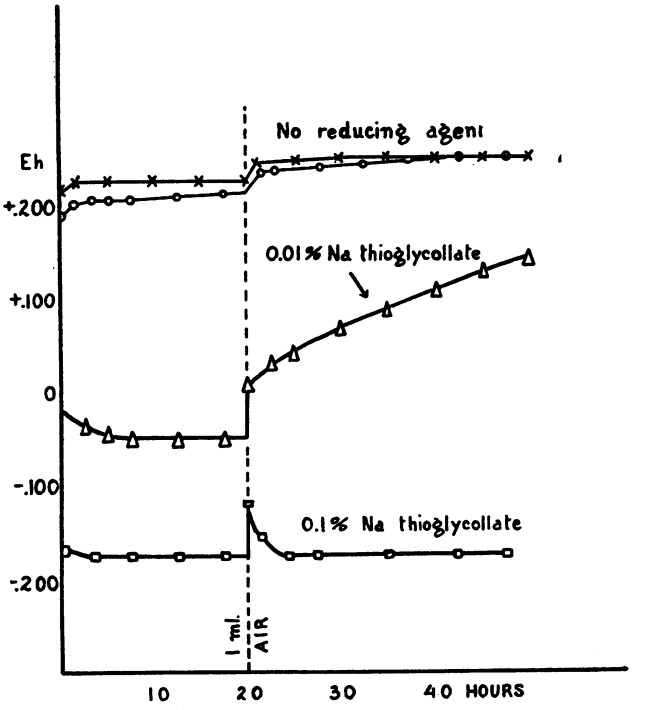

FIG. 2. GRAPHS INDICATING THE POISING EFFECT OF VARIOUS CONCENTRATIONS OF SODIUM THIOGLYCOLLATE IN PEPTONE BROTH
One ml. of air was rapidly bubbled through the media 20 hours after autoclaving. Ordinates represent Eh and abscissa time.

tends to produce slightly more negative levels. This substance produces a bleaching effect on the yellow pigment of the peptone.

(c) *Sugars as reducing agents.* When glucose is added to the peptone-phosphate-agar medium potential conditions resemble those developed as a result of the addition of thioglycollate, cysteine, etc. This is indicated in figure 3. From the left hand portion of the curves (fig. 3), it is apparent that the medium without sugar and without thioglycollate exhibited a strongly positive potential, and became more positive until equilibrium was reached in about 5 hours, at Eh +0.250 volt (curve A). Media containing glucose, in sharp contrast, exhib-

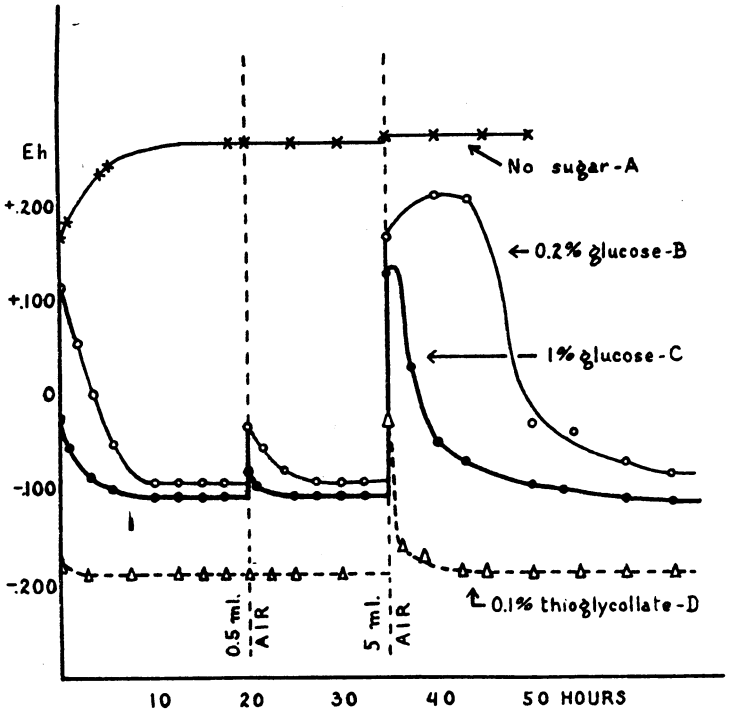

FIG. 3. GRAPH INDICATING THE INFLUENCE OF GLUCOSE ON THE O/R POTENTIALS AND POISING EFFECTS IN PEPTONE BROTH

0.5 and 5 ml. of air was rapidly bubbled through the media at 20 and 35 hours after autoclaving. Ordinates represent Eh and abscissa time.

ited an initial Eh of +0.100 to 0. The 0.2 per cent concentration gradually became more negative until an equilibrium was reached after about 10 hours at an Eh of very nearly −0.100 volt (curve B, fig. 3) and the 1.0 per cent came to about the same equilibrium in 5 hours (curve C, fig. 3). As in previous instances the medium with thioglycollate was at equilibrium from the start at Eh −0.180 volt. When sucrose was substituted for glucose, potentials remained essentially as when no sugar was present.

The poising effect of glucose is, however, very different from that of thioglycollate. This was determined by bubbling air through the media. After equi-

librium had been established for 10 to 20 hours, 0.5 ml. of air was introduced at the bottoms of the electrode vessels by introducing a fine capillary pipette, attached to a 0.5 ml. bulb, to the bottom of the vessels. A quick pressure of the bulb released a rapid stream of fine air bubbles. This caused a positive jump in the Eh in the 0.2 per cent glucose media to −0.030 volt followed by a negative drift to the original level in 6 to 8 hours (curve B, fig. 1). In contrast the 1.0 per cent sugar medium was influenced by the air to a lesser degree and the thioglycollate medium was not measurably influenced.

After another 10 to 30 hours of equilibrium 5 ml. of air was introduced by the same method. Again the 0.2 per cent sugar medium was thrown up to a strongly positive potential (curve B, fig. 3), followed by a slow negative return to the original negative level after some 30 hours. This larger amount of air also caused a marked positive potential jump in the 1.0 per cent glucose medium followed by a more rapid negative movement to the original level (curve C, fig. 3). In the thioglycollate medium, this amount of air caused a considerable positive potential jump though much less than in the sugar media, followed by a rapid return to the strongly negative Eh (curve D, fig. 1).

The media without glucose or thioglycollate or with sucrose, already fully oxidized, are not influenced by the introduction of these small amounts of air.

(d) *Influence of agar on O/R potentials.* The extent to which agar influences the O/R potential is suggested by figures 4 and 5. As previously shown, the peptone-phosphate broths with 0.1 per cent agar have a strongly positive Eh (fig. 4, upper curve). But in the medium containing 0.1 per cent sodium thioglycollate and agar, the Eh is negative and remains negative for the period of the experiment (fig. 4, lower curve). A portion of the same thioglycollate medium, lacking agar, gives a primary negative Eh but it gradually drifts to a positive potential until after 30 to 40 hours the Eh approximates that of the medium without added reducing agent (fig. 4, middle curve). Apparently the free diffusion of atmospheric oxygen, in the absence of agar, results in the slow oxidation of the thioglycollate.

Similarly, when 0.2 per cent glucose is added to the phosphate-peptone medium, without agar, the Eh remains strongly positive (fig. 5), but when the sugar is added to a portion of the same medium containing 0.1 per cent agar, there is a gradual negative drift, lasting for 25 to 30 hours, to an Eh of −0.80 volt.

The most effective concentration of agar is suggested by figure 6. A batch of the peptone-phosphate medium containing 0.2 per cent glucose was divided into several portions and agar added to provide concentrations from 0.02 to 0.25 per cent. It is apparent from curves A and B (fig. 5), that 0.02 per cent agar has very little influence on the O/R potential. In contrast, curves C to E (fig. 5), indicate that in the presence of 0.05 to 0.25 per cent agar the glucose reduces the medium to the same O/R potential. Equilibrium is reached more rapidly with the larger concentrations of agar. For many purposes the 0.1 per cent is most desirable as this is approximately the largest amount which can be added without producing a gel structure.

It therefore seems evident that the combination of sodium thioglycollate and

agar provides a well poised system which maintains a negative potential level even in the face of some mixing with air. Glucose, and presumably other reduc-

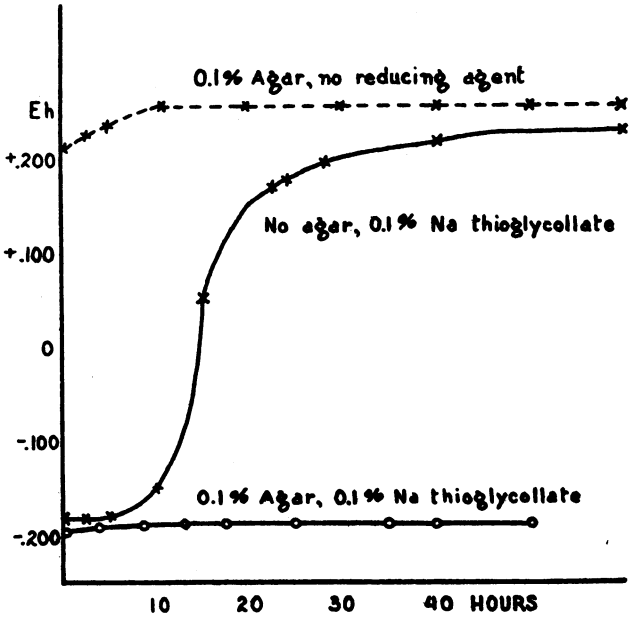

FIG. 4. GRAPHS INDICATING THE INFLUENCE OF 0.1 PER CENT AGAR ON THE O/R POTENTIAL OF PEPTONE BROTH IN THE PRESENCE AND ABSENCE OF 0.1 PER CENT SODIUM THIOGLYCOLLATE
Ordinates represent Eh and abscissa time

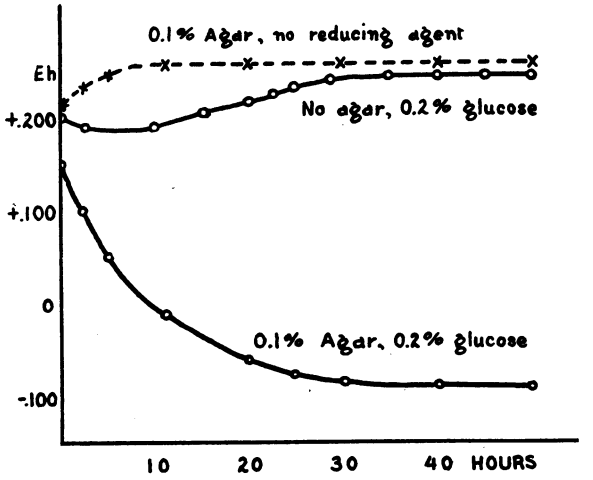

FIG. 5. GRAPHS INDICATING THE INFLUENCE OF 0.1 PER CENT AGAR ON THE O/R POTENTIAL OF PEPTONE BROTH IN THE PRESENCE AND ABSENCE OF 0.1 PER CENT GLUCOSE
Ordinates represent Eh and abscissa time

ing sugars, in the presence of agar provide a relatively poorly poised but definitely negative potential. It is also evident that the retardation of air diffusion does

not provide a negative O/R potential in this medium, unless a reducing substance is present.

The new Difco experimental anaerobic medium which contains 0.5 per cent of glucose and 0.1 per cent of agar, gives an Eh of approximately −0.100 volt. This too is undoubtedly due to the combined action of the glucose and agar. The excellent growth of all species of *Clostridium* which have been tried in this medium, as in the case of the simpler peptone-phosphate medium, indicate that special reducing agents are not essential for anaerobic growth in media *where reducing sugars can be used.*

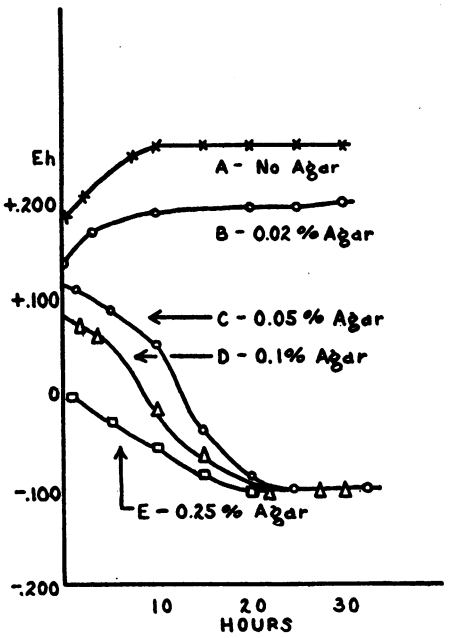

FIG. 6. GRAPHS INDICATING THE EFFECT OF INCREASING CONCENTRATIONS OF AGAR IN PEPTONE MEDIUM CONTAINING 0.2 PER CENT GLUCOSE
Ordinates represent Eh and abscissa time

2. O/R POTENTIAL OF GROWING CULTURES OF CLOSTRIDIA

Figure 7 indicates O/R potential changes in cultures of *Clostridium welchii* in sugar-free peptone-phosphate-agar medium with and without 0.1 per cent sodium thioglycollate. In this experiment a relatively large inoculum, 0.01 ml. of an 18-hour culture, was introduced into the medium without thioglycollate at the bottom of the electrode vessel with a Pasteur pipette. As in the former O/R potential determinations no correction for pH change has been attempted. In the sterile media there was little or no pH change but in cultures in sugar-free broth the change is from the initial pH 7.6 to approximately 6.5.

It is apparent that in the medium without thioglycollate, and with a strongly positive O/R potential, the introduction of the inoculum causes an immediate negative potential movement. Since this negative drop in potential begins be-

fore any significant amount of growth could take place the initial change must result from the reducing action of the inoculum, in the poorly poised system. Growth is perceptible by a clouding of the medium in 5 to 6 hours but the negative drift continues for 25 to 30 hours. In sharp contrast, in the thioglycollate-containing medium the inoculum has no influence on the Eh and a measurable change does not appear until some hours after growth is perceptible by a clouding of the medium. When the negative drift does begin it is more gradual and

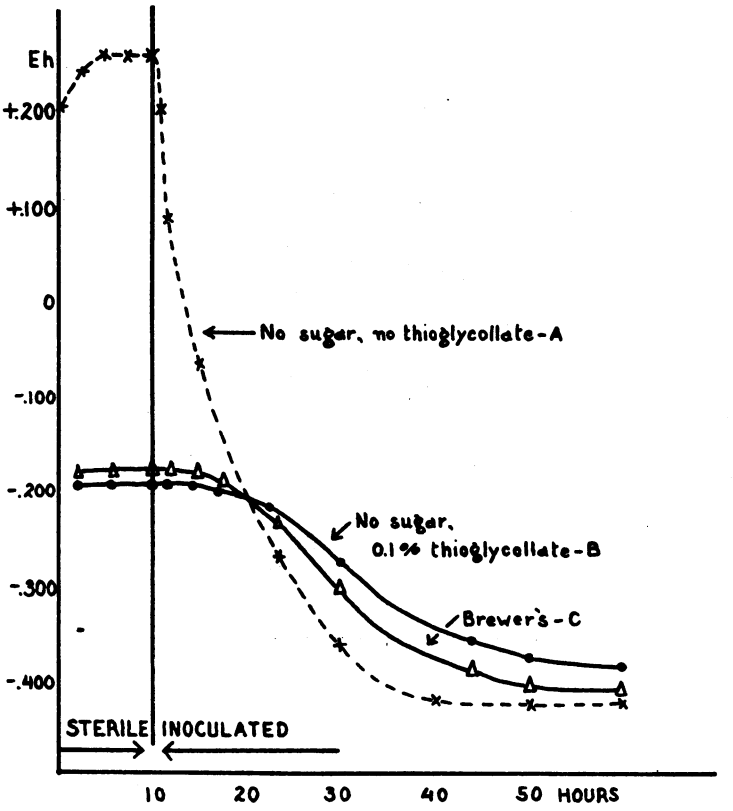

FIG. 7. GRAPHS INDICATING THE O/R POTENTIAL OF GROWING CULTURES OF *C. welchii* IN PEPTONE-AGAR MEDIUM WITH AND WITHOUT SODIUM THIOGLYCOLLATE AND IN BREWER'S MEDIUM
Ordinates represent Eh and abscissa time

the final Eh level is less negative than in the medium without thioglycollate. This, apparently, is the result of the poising action of the thioglycollate. Cysteine, ascorbic acid and sodium formaldehyde sulfoxylate have a similar influence on the growth-potential of *C. welchii* in this medium.

Brewer's medium which contains pork infusion and the same concentration of thioglycollate shows essentially the same potential change with the growth of *C. welchii* (curve C, fig. 7).

Smaller inocula result in a less precipitous negative drift in potential in the

medium without thioglycollate. Still smaller inocula produce no potential change and fail to initiate growth, as discussed in the next section. In the media containing thioglycollate, inocula down to the minimum necessary to start growth produce changes essentially as shown in curves B and C (fig. 7).

3. MINIMUM INOCULUM

In order to correlate the O/R potential of these media with their ability to support growth of various species of *Clostridium* a determination has been made of the minimum inoculum to initiate growth. All media were prepared, as previously described for potential determinations (in most instances the same lots of media were used for both potential and growth studies) and tubed in 9 ml. amounts in $\frac{5}{8}$-inch tubes. This gave the same depth of media in the small tubes as in the electrode vessels. The organisms to be tested were grown for 18 hours in the peptone-phosphate-agar medium without sugar and without thioglycollate or other reducing agent. This was made possible by the use of large inocula. The cultures were diluted serially in recently boiled 0.85 per cent NaCl solutions containing 0.1 per cent gelatin. One-tenth ml. amounts of the dilutions were delivered at the bottom of the tube of media to be tested without mixing beyond that resulting from the introduction of the pipette. Just before inoculation the tubes were held in a boiling water bath for 15 minutes and rapidly cooled in an ice bath.

(a) *Influence of sodium thioglycollate.* The peptone-phosphate-agar media, as described in Section 1(a), with 0.2 per cent glucose but no other added reducing agent has an initial Eh of −0.080 to −0.100 volt (fig. 3). As indicated in the first column of table 1, this medium supports good growth of all the species tested but a relatively large inoculum is required. The figures represent average results. There is, however, considerable variation in the effective minimum inoculum; several tubes from the same batch inoculated from the same serial dilution show variation. This is to be expected from the O/R potential data shown in figure 3. While the undisturbed medium assumes an Eh of −0.080 to −0.100 volt it is so poorly poised (fig. 3) that the stirring occasioned by introducing the inoculum may produce marked positive potential change.

It might be expected from figure 3 that a larger concentration of glucose, which more effectively poises the medium at a slightly more negative Eh, would provide a more stable medium in respect to inocula necessary to initiate growth.

The peptone-phosphate-agar with 0.1 per cent sodium thioglycollate and poised at Eh −0.180 to −0.200 volt (fig. 3), is effectively inoculated with much smaller amounts of all the cultures tested (second column, table 1).

Brewer's medium which contains agar and the same amount of sodium thioglycollate as the peptone-phosphate-agar medium and has the same initial Eh is inoculated with approximately the same minimum inoculum. This is in agreement with the results obtained by McClung (1940).

(b) *Influence of glucose and agar.* In order to test the influence of agar and glucose on the minimum inoculum the basic peptone-phosphate medium was made up without agar, glucose or other reducing agent. This was divided into

four portions and the following additions made: (*a*) no additions; (*b*) 0.1 per cent agar; (*c*) 0.2 per cent glucose; (*d*) 0.2 per cent glucose and 0.1 per cent agar, Eh +0.150 to +0.050; (*e*) 0.1 per cent sodium thioglycollate; (*f*) 0.1 per cent sodium thioglycollate and 0.1 per cent agar. The minimum inoculum to initiate growth,

TABLE 1

Minimum inoculum for growth of several gas gangrene and related Clostridia in media with and without sodium thioglycollate

Figures indicate minimum volume of 18-hour culture to initiate growth in 9 ml amounts of the three media.

	PEPTONE-PHOSPHATE-GLUCOSE-AGAR		BREWER'S WITH Na THIOGLYCOLLATE Eh −0.180 to −0.200
	No added reducing agent, Eh −0.080 to − 0.100	With Na thioglycollate, Eh −0.180 to −0.200	
C. aerofoetidum	10^{-4}	10^{-8}	10^{-8}
C. capitovalis	10^{-1}	10^{-5}	10^{-5}
C. carnis	10^{-2}	10^{-8}	10^{-8}
C. difficile	10^{-2}	10^{-5}	10^{-5}
C. fallax	10^{-4}	10^{-7}	10^{-6}
C. histolyticum	10^{-4}	10^{-8}	10^{-8}
C. novyi	10^{-3}	10^{-8}	10^{-8}
C. paraputrificum	10^{-1}	10^{-6}	10^{-6}
C. septicum	10^{-1}	10^{-7}	10^{-7}
C. sordellii	10^{-2}	10^{-5}	10^{-5}
C. sporogenes	10^{-2}	10^{-8}	10^{-8}
C. tetanomorphum	10^{-1}	10^{-6}	10^{-6}
C. tertium	10^{-4}	10^{-7}	10^{-7}
C. welchii	10^{-4}	10^{-8}	10^{-8}
C. tetani	10^{-1}	10^{-8}	10^{-8}

TABLE 2

	NO SUGAR OR OTHER REDUCING AGENT		0.2% GLUCOSE, NO OTHER REDUCING AGENT		0.1% SODIUM THIOGLYCOLLATE	
	No agar, Eh +0.200 to +0.250	0.1% agar, Eh +0.200 to +0.250	No agar, Eh +0.200 to +0.225	0.1% agar, Eh −0.080 to −0.100	No agar, Eh −0.150 to −0.180	0.1% agar, Eh −0.180 to −0.200
C. welchii	nil	10^{-2}	10^{-2}	10^{-4}	10^{-4}	10^{-8}
C. septicum	nil	nil	nil	10^{-3}	10^{-2}	10^{-7}
C. novyi	nil	10^{-1}	nil	10^{-4}	10^{-4}	10^{-8}
C. sporogenes	nil	10^{-1}	nil	10^{-3}	10^{-2}	10^{-7}
C. sordellii	nil	nil	nil	10^{-3}	10^{-3}	10^{-7}
C. tetani	nil	nil	nil	10^{-2}	10^{-2}	10^{-8}

of some six species of *Clostridium*, in the six media was then determined. Results are summarized in table 2.

From the first and second columns (table 2), it is apparent that this medium without glucose or other reducing agent, in the presence or the absence of agar, Eh +0.200 to +0.250, is not suitable for these species of *Clostridium*. The

basic medium with glucose but without agar, Eh +0.200 to +0.225 (column 3, table 2) is equally unsuitable. The addition of both glucose and agar results in a medium, Eh −0.080 to −0.100, which supports growth of all six species provided a moderately large inoculum is used. These results, however, are highly variable, apparently due to the indifferent buffering (fig. 3), and varying Eh resulting from inadvertent stirring with the inoculating pipette. These results contrast rather sharply with those obtained in the same medium poised with sodium thioglycollate. In the absence of agar (column 5, table 2), fair but somewhat variable results were obtained, evidently again due to poor poising (fig. 4). The thioglycollate plus agar gives, as previously shown, highly satisfactory results.

(c) *Explanation of large inocula.* It is a familiar observation that in certain media many species of bacteria will grow only from large inocula. It has been suggested by Clark (1924), Quastel and Stephenson (1926), and Fildes (1929) that in case of anaerobes this may be due to a localized negative drift in potential caused by the organisms of the inoculum.

In one series of experiments short spirals of platinum wire were sealed in the bottom of 15 ml. conical centrifuge tubes to serve as electrodes. When these tubes were filled with peptone-phosphate medium, without agar or reducing agent, and heavily inoculated with *C. welchii* the Eh dropped from Eh +0.225 volt to +0.025 volt in one hour. But when similarly inoculated tubes were centrifuged at high speed for half an hour, resulting in most of the organisms of the inoculum being packed about the electrodes, the O/R potential dropped from Eh +0.225 to Eh −0.200 in the same period. This is in agreement with Fildes' (1927) suggestion that clumps of *Clostridium tetani* spores may effect a localized negative drift in potential.

DISCUSSION

The results presented in this paper are in agreement with the principle that a favourable O/R potential is essential for the proliferation of an organism. It is shown that the optimum Eh for fifteen species of the genus *Clostridium* is in the vicinity of −0.200 volt.

A low concentration of glucose, and presumably of other reducing sugars, will slowly bring a slightly alkaline peptone solution to approximately this level but such a medium is very poorly poised and a slight admixture of air causes a pronounced positive drift in the potential. The presence of agar in small concentrations, the order of 0.1 per cent, greatly reduces the rate of diffusion of air and as a result stabilizes the potential.

More actively reducing substances, sodium thioglycollate, cysteine, ascorbic acid, sodium formaldehyde sulfoxylate produce a more negative O/R potential and a much more effective poising of the peptone medium than glucose. The addition of small concentrations of agar to the media containing one of these reducing agents results in a well poised negative O/R potential within the optimum range for the growth of most species, possibly all species, of pathogenic clostridia.

In a poorly poised medium organisms introduced as inoculum tend to produce a local area of reducing potential favourable to growth.

SUMMARY

1. Some fifteen species of pathogenic clostridia grow luxuriantly from small inocula in a simple, slightly alkaline, peptone solution provided it is poised at a favourable O/R potential.

2. The optimum Eh for these species is in the region of −0.2 volt.

3. Glucose, in this medium, produces an O/R potential, though poorly poised, which approximates the optimum for these species.

4. Sodium thioglycollate, cysteine, ascorbic acid, sodium formaldehyde sulfoxylate produce better poised O/R potentials nearer the optimum for pathogenic clostridia than is produced by glucose.

5. The addition of small amounts of agar to the medium greatly increases the stabilization of the O/R potentials.

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