THE INFLUENCE OF VITAMIN C ON THE GROWTH OF ANAEROBES IN THE PRESENCE OF AIR, WITH SPECIAL REFERENCE TO THE RELATIVE SIGNIFICANCE OF EH AND O_2 IN THE GROWTH OF ANAEROBES

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INTRODUCTION

The problem of biotic activity in the absence of free oxygen has occupied the attention of physiologists and bacteriologists since the days of Pasteur. Pasteur (1861) postulated that free O_2 was lethal to anaerobic bacteria. This view has been contested by McLeod and Gordon (1923) who claimed that the toxic effect was due to peroxides; according to these authors the obligate anaerobes were devoid of catalase and hence could not destroy the peroxides formed by autooxidizable substances. This view was supported by Callow (1923) and received further support from the findings of Avery and Morgan (1924) that anaerobes grew freely under aerobic conditions if unheated, sterile, plant extracts were added to broth. The explanation advanced by these authors was that the plant catalases and peroxidases destroyed the peroxides formed and thus enabled the anaerobes to continue their growth.

This hypothesis was, however, rendered untenable by the findings of Novy (1925) and Sherman (1926). Novy showed that anaerobic growth occurred in one arm of an H tube if a piece of unsterilized potato or a culture of *Bacillus subtilis* was kept in the other arm; in other words, growth was possible when the free O_2 was removed from the tube. Sherman demonstrated that anaerobic propionic acid bacteria did produce catalase.

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And finally Quastel and Stephenson (1926) showed that H_2O_2 does not necessarily inhibit the growth of anaerobes under all conditions. These authors cultivated *Clostridium sporogenes* in the presence of small amounts of H_2O_2 in media containing 0.1 per cent cystein, glutathion or thioglycocol which lowered the reduction potential even in the presence of H_2O_2. Hosoya and Kishino (1925) obtained similar results with cystein broth (0.001 per cent cystein-hydrochloride) and Frei and Riedmueller (1930) with cystein-agar (1.0 per cent cystein in a 2.5 per cent agar).

More recently a number of investigators have pointed out the importance of the reduction potential in anaerobic growth. Coulter (1928), Dubos (1929), Aubel, Aubertin and Genevois (1929), Knight (1930), Plotz and Geloso (1930), Lepper and Martin (1930) have measured the reduction potential either by the potentiometer or by indicators. Plotz and Geloso (1930) have established that the minimum reduction potential for anaerobic growth is Eh +0.036 v. and Fildes (1929) and Fildes and Knight (1930) put it at Eh +0.11 v. Lepper and Martin showed that the Eh of the meat medium used for anaerobes is colorimetrically −0.2 v. and potentiometrically −0.174 v.

This view as to the importance of the reduction potential in the growth of anaerobes has been contested by Knaysi and Dutky (1934, 1936). On the basis of their investigations these authors concluded that the significant factor is the partial O_2 pressure, and that the reduction potential may merely serve as an indicator of the oxygen tension. They obtained good growth of *Clostridium butyricum* at low oxygen tension although the Eh was raised by the addition of potassium ferricyanide to +0.335 v.; on the other hand the same organism failed to grow when the O_2 tension was regulated so that the Eh of the medium was +0.3 v.

During the last two years we have been studying in this laboratory the effect of vitamin C on toxin production by *Corynebacterium diphtheriae* (Kligler 1936), as well as on viruses (Kligler and Bernkopf 1937). Since ascorbic acid belongs to the class of highly reducing substances and is at the same time

an important constituent of the plant and animal organism, we undertook a study of its effect on the growth of anaerobes.

In this paper we present the results obtained with *Clostridium welchii*.[2]

EXPERIMENTAL

I. The minimal amount of vitamin C required for "aerobic" growth of Clostridium welchii

The following medium was used in these experiments:

Meat extract	3.0 grams
Peptone	10.0 grams
NaCl	10.0 grams
K_2HPO_4	2.0 grams
H_2O	1000 grams
Reaction	pH 7.2–7.4

The medium was prepared in the usual manner, filtered, distributed in tubes and autoclaved. The vitamin C solution was made in distilled water and filtered through a Seitz filter. It was prepared fresh each time and desired amounts added to the broth just before inoculation with the culture. A 24-hour meat-broth culture was used for inoculation; 0.05 cc. of the liquid culture was added to each tube. The tubes were stoppered with cotton in the usual way.

Each experiment was repeated several times with the same results. We shall, therefore, present here only the results of type experiments.

The effect of various concentrations of vitamin C in broth on the growth of the culture is shown in table 1. It will be noted that growth occurred in the tubes containing 0.02 per cent but not in the tubes containing 0.01 per cent vitamin. When growth occurred, its intensity was the same in all tubes.

A similar series of experiments was made with semi-solid agar. This medium was prepared by the addition of 0.4 per cent agar to the stock broth used in the preceding experiment.

[2] An abstract of a somewhat similar investigation on the effect of vitamin C on anaerobic growth by Ehrisman (1936) came to our attention when our own experiments were concluded.

The results are shown in table 2. It will be noted that in this medium a much smaller amount of vitamin C sufficed for growth.

In plain broth the minimal amount of ascorbic acid for growth was 0.02 per cent while in the semi-solid agar it was 0.03 per thousand. It is also noteworthy that, whereas in the broth the turbidity is uniform in the entire tube, in the semi-solid agar growth stops short about 0.5 cm. from the top.

TABLE 1

Minimal concentration of vitamin C for growth of C. welchii in broth

CONCENTRATION OF VITAMIN C (PARTS PER THOUSAND)	DENSITY OF GROWTH (24 HOURS AT 37°C.)
2.0	++
1.0	++
0.5	++
0.2	++
0.1	−
0.05	−

TABLE 2

Minimal amount of vitamin C for growth of C. welchii in semi-solid agar

CONCENTRATION OF VITAMIN C (PARTS PER THOUSAND)	NATURE AND INTENSITY OF GROWTH
0.2	Upper 0.5 cm. sterile; lower part ++
0.1	Upper 0.5 cm. sterile; lower part ++
0.05	Upper 0.5 cm. sterile; lower part ++
0.03	Upper 0.5 cm. sterile; lower part ++
0.02	No growth
0.01	No growth

We next studied the effect of glucose. The media contained varying concentrations of glucose; the fluid media contained 1 part and the semi-solid media only 0.2 parts of vitamin C per thousand. The results are shown in table 3.

There was no growth in media containing 1.0 per cent glucose and only 0.1 per thousand vitamin.

It is apparent that glucose does not effect the concentration of vitamin C required for growth. It serves as a nutrient and hence better growth is obtained when it is present in sufficient amount.

Peptone proved to perform a different function. Simple media were prepared consisting of Tyrode solution with varying concentrations of peptone. The results are shown in table 4. As can be seen from the table, no growth occurred in tubes

TABLE 3

The effect of glucose on the "aerobic" growth of C. welchii in media containing vitamin C

MEDIA	NATURE OF GROWTH
Broth + 1 per thousand vitamin C; + 0 glucose	++
Broth + 1 per thousand vitamin C; + 0.05 per cent glucose	++
Broth + 1 per thousand vitamin C; + 0.1 per cent glucose	++
Broth + 1 per thousand vitamin C; + 0.5 per cent glucose	+++
Broth + 1 per thousand vitamin C; + 1.0 per cent glucose	+++
Broth + 1.0 per cent glucose	−
Semi-solid + 0.2 per thousand vitamin C. + 0.0 per cent glucose	++*
Semi-solid + 0.2 per thousand vitamin C. + 0.5 per cent glucose	+++*
Semi-solid + 0.2 per thousand vitamin C. + 1.0 per cent glucose	+++*
Semi-solid + 1.0 per cent glucose	−†

* No growth 0.5 cm. from top.
† No growth.

TABLE 4

Effect of varying concentrations of peptone on the minimum concentration of vitamin C required for "aerobic" growth of C. welchii

CONCENTRATION OF VITAMIN C (PARTS PER THOUSAND)	CONCENTRATIONS OF PEPTONE (PER CENT)									
	0.0	0.25	0.5	1.0	1.5	2.0	2.5	3.0	4.0	5.0
1.0	−	±	+	+	++	++	++	++	+++	+++
0.2	−	−	±	+	+	++	++	++	++	+++
0.05	−	−	−	−	−	+	++	++	++	+++
0.02	−	−	−	−	−	−	−	−	−	−
0.00	−	−	−	−	−	−	−	−	−	−

−, ±, +, ++ = intensity of growth.

containing 0.02 per thousand vitamin C, no matter how high the concentration of peptone. However, above this amount there is a definite relation between the concentration of peptone and vitamin C; the greater the concentration of peptone, the less vitamin C is required. The intensity of growth also increases

with the concentration of peptone. Peptone serves, therefore, a double function; it provides reducing substances as well as nutrients.

II. Fate of vitamin C in inoculated and uninoculated media

It was naturally of interest to ascertain what happened to the vitamin C and in what way it exerted its influence on the

TABLE 5

Loss of vitamin C in inoculated and uninoculated broth containing glucose

CONCENTRATION OF GLUCOSE	INOCULATED	GROWTH	AMOUNT OF VITAMIN C	LOSS OF VITAMIN C	
(a) 5 mgm. vitamin C per 5 cc. medium = 1 per thousand; findings after 24 hours					
per cent			*mgm.*	*mgm.*	*per cent*
0.0	−	−	2.8	2.2	44.0
0.0	+	++	3.6	1.4	28.0
0.05	−	−	2.8	2.2	44.0
0.05	+	++	3.2	1.8	36.0
0.10	−	−	2.8	2.2	44.0
0.10	+	++	3.6	1.4	28.0
0.50	−	−	2.7	2.3	46.0
0.50	+	+++	3.4	1.6	32.0
(b) 10 mgm. vitamin C per 5 cc. = 2 per thousand					
0.0	−	−	7.1	2.9	29.0
0.0	+	++	8.4	1.6	16.0
0.05	−	−	7.2	2.8	28.0
0.05	+	++	8.4	1.6	16.0
0.10	−	−	7.2	2.8	28.0
0.10	+	++	8.7	1.3	13.0
0.50	−	−	7.3	2.7	27.0
0.50	+	+++	8.6	1.4	14.0

cultures. Meat-extract broth containing varying concentrations of glucose and vitamin C was used. Parallel tubes, one inoculated and the other uninoculated, were incubated at 37° for 24 hours. At the end of this period the content of vitamin C was determined by titration with the Tillmans reagent. The results are shown in table 5.

It is interesting to note that the actual loss in the amount of vitamin C is the same in media containing 2:1000 as in those

containing 1:1000 of vitamin. The actual loss in milligrams in 5 cc. was 2.2 to 2.9. Whether this loss is due to oxidation or to combination with substances in the media is now under investigation (Leibowitz and Guggenheim). Another point of interest is the fact that the loss of vitamin was about twice as high in the uninoculated as in the inoculated tubes. It is evident that bacterial growth inhibits the loss, possibly as a result of the re-

TABLE 6

Influence of peptone on the loss of vitamin C in media containing

CONCENTRATION OF PEPTONE	INOCULATED	GROWTH	AMOUNT OF VITAMIN C	LOSS OF VITAMIN C	
(a) 5 mgm. vitamin per 5 cc. = 1 per thousand; findings after 24 hours					
per cent			*mgm.*	*mgm.*	*per cent*
0.0	Control	−	1.25	3.75	75.0
0.5	−	−	2.1	2.9	58.0
0.5	+	±	2.3	2.7	54.0
1.0	−	−	2.5	2.5	50.0
1.0	+	+	2.5	2.5	50.0
2.0	−	−	2.6	2.4	48.0
2.0	+	++	2.6	2.4	48.0
4.0	−	−	2.7	2.3	46.0
4.0	+	+++	2.7	2.3	46.0
(b) 10 mgm. vitamin C per 5 cc. = 2 per thousand					
0.0	Control	−	4.8	5.2	52.0
0.5	−	−	5.1	4.9	49.0
0.5	+	±	5.3	4.7	47.0
1.0	−	−	6.9	3.1	31.0
1.0	+	+	7.0	3.0	30.0
2.0	−	−	7.1	2.9	29.0
2.0	+	++	7.1	2.9	29.0
4.0	−	−	7.0	3.0	30.0
4.0	+	+++	7.1	2.9	29.0

ducing substances produced by the bacteria. Glucose exerts no influence on the vitamin either in the inoculated or uninoculated tubes.

Similar analyses were made of media containing peptone. These media consisted of Tyrode solution to which were added various concentrations of vitamin and peptone respectively. The results are shown in table 6.

These results differ from those obtained in the glucose media. Whereas glucose has no effect on the vitamin, peptone exercises a protective influence on vitamin C. The loss of the vitamin in the media decreases with increasing amounts of peptone up to 1 per cent; beyond that point no difference is noted. However, in contrast with the glucose-broth, the growth of bacteria in the tyrode-peptone media does not modify the rate of loss of vitamin C. There is apparently a limiting protective or sparing effect and in peptone media this is exerted by the peptone. It is also evident that the bacteria do not use vitamin C as a nutriment.

These results complement those reported earlier in this paper on the influence of glucose and peptone on the vitamin C minimum for growth. Glucose which, as shown by subsequent experiments, does not influence the reduction potential, also fails to prevent oxidation of vitamin C; it improves growth only when a sufficient amount of vitamin C is present to make growth possible. Peptone, on the other hand, which lowers the reduction potential, protects vitamin C and permits growth, within certain limits, even in the presence of smaller concentrations of vitamin C. The vitamin obviously serves the function of a catalyzer.

III. Determination of reduction potential

Having established the conditions favouring the growth of anaerobes in the presence of air, we proceeded to determine whether the reduction potential or the oxygen concentration was the determining factor in the growth of anaerobes. The measurements of the reduction potential were made colorimetrically with indigo-carmin. To every 5 cc. of the medium we added 0.1 cc. of a 0.5 per cent solution of the indicator. The media were not freed of air, since we were concerned with the reduction potential under conditions under which growth occurred. The color change which occurred during 24 hours was determined in the usual way in a colorimeter.

The results are given in tables 7 and 8. It is apparent that the intensity of reduction parallels the increasing concentrations

of vitamin. Glucose does not influence the reduction. It is also of interest to note that the minimum amount of vitamin making growth possible, i.e., 0.2 per thousand causes a reduction of about 75 per cent, equivalent to an Eh −0.125 v. Growth does not occur when the amount is such that the reduction of indigo-carmin is less than 75 per cent.

TABLE 7

Reduction of indigo-carmin in media containing various concentrations of vitamin, with and without glucose (Basic medium beef-extract broth)

CONCENTRATION OF VITAMIN	CONCENTRATION OF GLUCOSE	REDUCTION OF INDICATOR	GROWTH
parts per thousand	*per cent*	*per cent*	
2.0	0.0	100	+
1.0	0.0	90	+
0.5	0.0	75	+
0.1	0.0	50	−
1.0	0.5	80	+
0.5	0.5	75	+
0.1	0.5	50	−

TABLE 8

Reduction of indigo-carmin in media containing various concentrations of vitamin C and peptone in Tyrode solution

CONCENTRATION OF VITAMIN	CONCENTRATION OF PEPTONE (PER CENT)								
	0	0.25	0.5	1.0	1.5	2.0	3.0	4.0	5.0
parts per thousand									
1.0	0*	75	90	90	90	90	90	100	100
0.2	0	50	75	75	75	90	90	90	90
0.05	0	25	50	50	50	75	75	75	75

* The figures represent per cent reduction. It should be noted that there is a limiting point where no increased reduction is obtained no matter how much peptone is added.

As was to be expected from the preceding experiments, peptone itself exerts a reducing effect. With increasing amounts of peptone, smaller quantities of Vitamin C are sufficient to give 75 per cent or more reduction of the indicator.

If table 8 is compared with table 4, it becomes apparent that

there is a close parallelism between the intensity of reduction and the ability of the organisms to grow. Growth occurred in all mixtures of vitamin-peptone which gave 75 per cent reduction of the indicator or an Eh of −0.125 v. Wherever the reduction was less, no growth occurred.

IV. The oxygen content of the Vitamin-C-containing media

In order to establish whether the free O_2 or the reduction potential was the determining factor in the growth of anaerobes, it

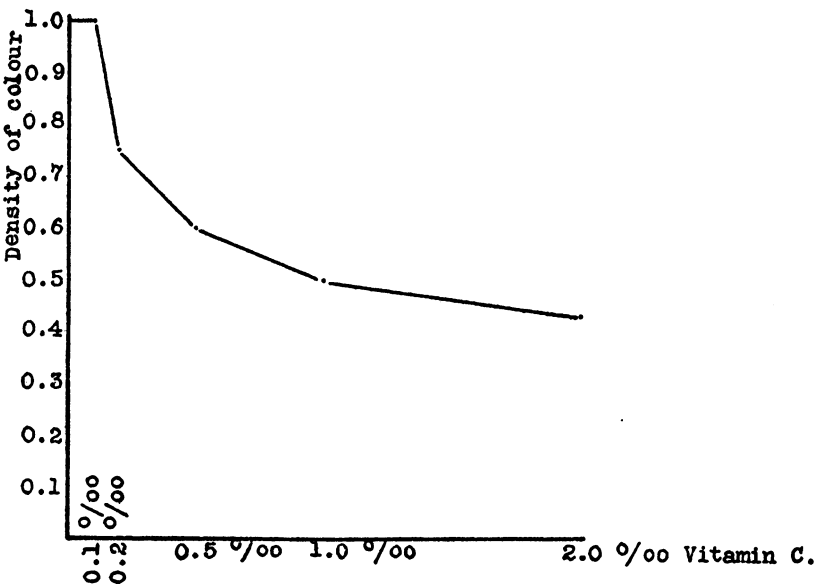

FIG. 1. EFFECT OF VITAMIN C ON O_2 CONTENT OF BROTH

was essential to ascertain whether, in addition to lowering the reduction potential, the vitamin also reduced the oxygen content of the medium by autooxidation. Pyrogallol was used to measure the oxygen in the medium. To every 5 cc. of broth, containing varying quantities of vitamin C, we added 0.1 cc. of a 2 per cent aqueous solution of pyrogallol and 0.1 cc. of a 5 per cent solution of NaOH; after 15 to 20 minutes the intensity of color was measured in a colorimeter, broth without vitamin C serving as the standard. Taking the color of the broth as 1.0,

it was possible to grade the shades of color in the tubes containing various concentrations of vitamin. The results are given in table 9 and figure 1.

It will be noted that considerable amounts of free oxygen were still present in the tubes in which growth occurred. In other words, the amount of vitamin C sufficient to make growth possible used up only a relatively small amount of the free oxygen.

In order to obtain some idea of the oxygen tension in the tubes supporting growth, the pyrogallol test described above was applied to broth under different oxygen tensions. The broth tubes were fitted with two-holed rubber stoppers containing glass capillaries and evacuated to varying degrees. After the

TABLE 9

The relative amount of free oxygen found in broth containing different amounts of vitamin C

CONCENTRATION OF VITAMIN (PARTS PER THOUSAND)	RELATIVE INTENSITY OF COLOR	GROWTH OF ANAEROBE, (C. WELCHII)
0.0 (control)	1.0	−
0.05	1.0	−
0.1	1.0	−
0.2	0.75	+
0.5	0.6	+
1.0	0.5	+
2.0	0.43	+

evacuation 0.1 cc. pyrogallol and 0.1 cc. NaOH were sucked into the tubes and the intensity of color measured in the manner described above. It was found that an air pressure of 675 mm. or a partial oxygen pressure of 135 mm. gave a color with pyrogallol equal to 0.71 of the standard. The relation of color density of the pyrogallol to O_2 tension is shown in figure 2. It will be noted that a reduction of only 3 mm. in the oxygen tension gives a color reaction at which growth occurred in the presence of vitamin C.

It is apparent, therefore, that growth of *Clostridium welchii* occurred in tubes with over 95 per cent of the normal oxygen tension, provided an adequate amount of vitamin C was present.

In other words, the significant function of vitamin C is to lower the reduction potential.

These results were confirmed in another way. Broth tubes containing varying amounts of vitamin C were stoppered with corks and left in the incubator overnight. The next day they were examined by the pyragallol method described above. All tubes gave a color density of 0.9 to 1.0. Apparently, during

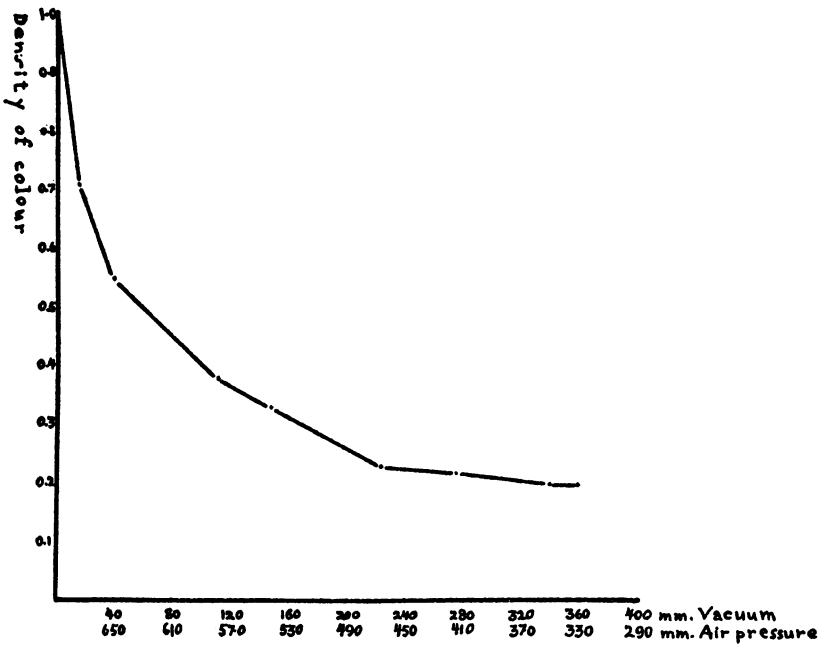

FIG. 2. RELATION OF O_2 CONCENTRATION TO COLOR DENSITY OF PYROGALLOL SOLUTION

the night the oxygen used up by the vitamin is replaced from the air in the tube, so that practically "aerobic" conditions existed even in tubes containing 2 parts per thousand of the vitamin.

Further evidence that the reduction potential was the decisive factor in the growth of *Clostridium welchii* under "aerobic" conditions was furnished by another type of experiment. In this experiment we added to all tubes 0.05 per thousand vitamin

C, an amount inadequate for growth in broth. The media were inoculated with *Clostridium welchii* and then the tubes were evacuated in varying degrees, the maximum being 315 mm. No growth occurred in any of the tubes although the pyrogallol test in the last tubes was 0.2 of the standard. It appears then that when the reduction potential was not suitable, no growth occurred, even when the O_2 tension was far below that found in tubes where good growth occurred.

DISCUSSION

The experiments reported above appear to us to clear up the moot point as to the determining factor in the growth of anaerobes—if *Clostridium welchii* is accepted as a type. It seems that anaerobiosis is determined by the reduction potential of the medium rather than by other factors. The favorable effect of plant juices is presumably due to the reducing substances present and not to the catalase and peroxidase as postulated by Avery and Morgan (1924).

It is not easy to establish the mechanism involved. But it may be assumed that the enzymic or rather catalytic system of anaerobes can only be active at a given reduction potential. At that potential, free O_2 does not interfere with its oxidation-reduction activity. At a point above that potential, the free oxygen interferes with the function of the anaerobes and growth is not possible. An illustrative experiment in this connection is reported by Wurmser (1925). This author showed that the synthesis of alanin from pyruvic acid and ammonia with glucose as H donator is possible only at an Eh less than 22; at a higher Eh the synthesis does not occur, the glucose reacts with the free oxygen, is oxidized and fails to act as an H donator.

This view enables us also to explain the results of Knaysi and Dutky, who obtained growth of anaerobes at a high reduction potential in the absence of oxygen. So long as oxygen is absent, there can be no interference with the cell oxidation-reduction processes. In the presence of O_2, however, these processes are possible only when the reduction potential is so low that O_2 interference is eliminated.

These experiments also suggest the function of Vitamin C in the organism. On the one hand, it serves as a catalyzer and makes possible reactions which are dependent on its powerful reducing properties. On the other hand, it is dependent on other reducing substances to prevent its own oxidation. It is this constant oxidation which necessitates the continual replacement of vitamin C.

SUMMARY

1. Addition of vitamin C to fluid media favors the growth of anaerobes (*Clostridium welchii*) in the presence of air.

2. Glucose improves growth but does not affect the minimal amount of vitamin required to make growth possible.

3. In tyrode-peptone media the amount of vitamin C required to make growth possible decreases (within limits) as the concentration of peptone is increased. If the concentration of vitamin C is below 0.05 per thousand, growth fails even if large amounts of peptone are added.

4. The reduction potential of the medium at which growth of *Clostridium welchii* occurred was about -0.125 v. The addition of glucose does not modify the potential and exerts no influence on the loss of vitamin C. The addition of peptone lowers the potential and exerts a protective effect on the vitamin added to the medium.

5. Vitamin C presumably acts as a catalyzer for anaerobes. It lowers the reduction potential of the media but does not appreciably affect the O_2 tension.

6. These experiments indicate that anaerobic growth is determined by the Eh and not by O_2 tension. Growth is possible in the presence of air when the Eh is sufficiently low. If the Eh is above the critical point, free O_2 interferes with the oxidation-reduction processes of the cell.

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