THE INITIATION OF GROWTH OF CERTAIN FACULTATIVE ANAEROBES AS RELATED TO OXIDATION-REDUCTION PROCESSES IN THE MEDIUM.

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The expression "value of a bacteriological medium" is a loose one; it probably covers a number of characteristics of that medium, many of which are perhaps unrelated. The following are examples of the points of view from which the medium may be considered in this relation.

1. Initiation of growth: (*a*) size of minimum inoculum required for growth to develop, (*b*) duration of the lag period.
2. Density of growth: number of cells developing per unit capacity of medium.
3. Viability of the cultures.

This paper will be limited to a survey of some of the conditions which affect the size of the inoculum required to intiate growth.

HISTORICAL.

It is a well known fact that the growth of organisms difficultly cultivable is rendered much easier—even in unfavorable media—by the use of large inocula. This phemonenon is probably related to what has been termed "allelocatalysis" by Robertson (1) in his studies on transplants of protozoa, and "communal activity," by Churchman and Kahn (2) in their investigations on the bacteriostatic action of certain dyes.

The work of Valley and Rettger (3) indicates that many organisms grow slowly or not at all until a certain minimum concentration of carbon dioxide is present in the medium; the suggestion has been made that the introduction of a large inoculum may hasten the production of a favorable concentration of CO_2.

Gillespie (4) observed that "it requires much smaller numbers of pneumococci to start growth on agar than are required to start a growth in broth." He found also that the same result as with agar could be obtained by seeding the pneumococci on a fragment of filter paper on the surface of the broth. According to him, "this phenomenon is probably dependent entirely on physical differences

in the two kinds of media, and bears some relation to the differences in possibilities for diffusion in the two media."

The possible relation of minimum inoculum to oxidation-reduction processes has been expressed several times in the literature. In the course of his studies on the physiology of *B. lepisepticum*, Webster (5) observed that an inoculum of at least 100,000 cells (per 5 cc. broth), was necessary for growth to develop under aerobic conditions. On the other hand, growth occurred with an inoculum of only a very few cells when the culture was incubated under anaerobic conditions, or in the presence of sterile blood.

Burnet (6) showed that nutrient agar plates, exposed to light, while still capable of growing staphylococcus when heavily seeded, did not allow growth of "isolated" organisms. ("Isolated" organisms were obtained by spreading over the plate a very dilute suspension of bacteria.) But such "isolated" organisms would grow in the neighborhood of colonies of the same or other organisms, obtained by local heavy seedings. Nutrient agar which had been exposed to light and then heated for 30 minutes at 100°C. gave normal growth, unless exposure to light had been extremely prolonged. The explanation offered was that exposure to light resulted in the formation of a peroxide-like substance with a bacteriostatic action; reducing substances, formed in the course of metabolism in the large colonies (obtained by local heavy seedings) diffused through the agar and reduced the peroxides, thus allowing growth of "isolated" organisms around the colonies. Destruction of the peroxide was also obtained by heating the medium. Working with an anaerobe, *B. sporogenes*, Quastel and Stephenson (7) observed that this organism could be grown under apparent "aerobic" conditions, in tryptic digested broth, by the use of a large inoculum. A very small inoculum was sufficient to initiate "aerobic growth" when 0.1 per cent cysteine was added to the broth. Reduced glutathione and thioglycollic acid were found to play the same rôle as cysteine. According to Quastel and Stephenson, the mechanism of the process is as follows: In the course of its metabolism, *B. sporogenes* produces reduced compounds giving the $-SH$ reaction; the object of a large inoculum is to introduce enough of these reduced sulphydryl compounds to provide reducing conditions in the broth. The addition of cysteine serves of course the same purpose.

W. M. Clark (8) had previously pointed out that actively growing anaerobic cells, if not overtaxed, can establish their own reducing conditions even in the presence of a certain amount of molecular oxygen.

Aubel and Aubertin (9) seeded different organisms into tubes of glucose agar and observed at what levels of the agar growth developed. By observing the reduction of indicators of oxidation-reduction potentials in sterile tubes of the same medium, and comparing the findings with the results of the growth experiments, they concluded that the life of strict anaerobes is possible only when the rH of the medium is lower than 12.

Before attempting to analyze the mechanism whereby the oxidation-reduction properties of the medium control the minimum inoculum required to initiate growth, it is important to realize that bacterial cells themselves possess active and independent oxidation-reduction systems. The manifestations and nature of these systems are especially well known in the case of Pneumococcus.

Avery and Neill (10) have described a number of different oxidation-reduction processes which are exhibited by cultures or sterile extracts of pneumococci; such are the consumption of molecular oxygen, the production of peroxide, the oxidation of hemotoxin, the oxidation of hemoglobin to methemoglobin, and the oxidative destruction of various endocellular enzymes. The same cultures or extracts of pneumococci also reduce methylene blue to methylene white and methemoglobin to hemoglobin when the system is kept under anaerobic conditions.

These active oxidation-reduction systems of the cells consist of two components: (1) a cellular thermolabile constituent which is not removed by washing, (2) thermostable autoxidizable substances which are lacking in washed cells and which are not necessarily of pneumococcus origin, since they may be supplied by muscle infusion and yeast extract.

It is probable that a complete understanding of the influence of oxidation-reduction processes in bacterial growth will require a study of the interrelations between the oxidation-reduction systems of the media and of the cells.

EXPERIMENTAL.

The influence of the nature of the medium and of environmental conditions on the size of the minimum inoculum giving rise to growth, was studied by the following technique.

Tubes containing 5 cc. of the medium under consideration were inoculated with varying amount of culture. These inocula are referred to as 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, ...10^{-8}, corresponding to 0.1....0.00000001 cc. of a 12 hour culture in plain broth.

The cultures used were: Pneumococcus S forms, Type I (1/200/5), II (D/39/40), III (A/66/64), and R forms derived from Types I (1/193/R), II (D/39/R), and III (M/3/R). Human strains of hemolytic streptococcus, L, S/43, S/23/Glossy, S/23/45 Matt. *Staphylococcus aureus.*

Inocula were never taken from blood cultures, but from the third transfer in plain broth in order to prevent the carrying over of some of the blood constituents into the medium to be tested.

These plain broth cultures were plated on blood agar to determine the number of organisms present in the inoculum. There was of course some variation, but in general, plates inoculated with 10^{-7} of plain broth cultures of Pneumococcus or human strains of *Streptococcus hæmolyticus* showed only a few colonies (1 to 10). With Staphylococcus, the last positive plates were obtained with 10^{-9} cc. inoculum.

The plain broth was prepared from meat infusion according to the standard method. Fairchild's peptone (lot 280630) was used in the preparation. Cysteine solutions were prepared from cysteine hydrochloride and autoclaved. As the neutralized solution oxidizes very rapidly, while the acid solution is more stable, the latter was neutralized with sterile NaOH only at the time of being used. "Anaerobic" conditions were provided by sealing the cultures with a 2 cm. layer of sterile vaseline.

TABLE I.

Growth of Pneumococcus and Streptococcus in the Presence of Different Concentrations of Blood.

Medium	Smallest inocula with which growth was obtained			
	Pneumococcus II		*S. hæmolyticus* (human strains)	
	"S" D/39/40	"R" D/39/R	L	S/43
Plain broth	10^{-2}	10^{-2}	10^{-2}	10^{-3}
Plain broth + 0.001 per cent blood	10^{-2}	10^{-2}	10^{-2}	10^{-2}
Plain broth + 0.01 per cent blood	10^{-4}	10^{-4}	10^{-5}	10^{-4}
Plain broth + 0.1 per cent blood	10^{-8}	10^{-8}	10^{-8}	10^{-7}
Plain broth + 1 per cent blood	10^{-7}	10^{-6}	10^{-6}	10^{-7}

I. Growth of Streptococcus and Pneumococcus in Blood Broth.

Blood broth is known to be an ideal medium for the growth of pneumococci and streptococci. The attempt was made to ascertain what concentration of blood is necessary to insure growth with the smallest possible inocula.

Experiment 1.—The plain broth used had been prepared 1 week before the test. Sterile rabbit blood was added to the medium in amounts sufficient to give final concentrations varying from 1 per cent to 0.001 per cent. Table I indicates, in cubic centimeters of plain broth cultures, the smallest inocula with which growth was obtained.

According to this experiment, blood is still active in a dilution of 0.01 per cent. It may be doubted whether the action of such a small amount

corresponds to the addition of some nutrient lacking in the broth. There have been suggestions that the action of the blood is due to the fact that it contains a peroxidase or a catalase and perhaps also the V factor which has been associated with yeast extract. It may be recalled here that Thjotta and Avery (11) found that two factors (X and V) are essential for the growth of certain strains of influenza bacillus. The factor V appears to be of the nature of a vitamine and can be supplied in the form of yeast extract; X was claimed to be an iron compound and hematin was used as its source in routine technique. X exhibits peroxidase and catalase activity. Both X and V are present in the blood.

Attempts have been made therefore to simulate the action of blood by the addition of certain iron compounds (possessing catalase, peroxidase, and oxidase activity) in the presence or in the absence of yeast extract.

II. The Influence of Iron Compounds on the Growth of Pneumococcus.

Experiment 2.—In these tests, a large number of iron compounds were used.[1] Among them may be mentioned.

Active Fe_2O_3 (Siderac)
Active Fe_2O_3 (Baudisch)
} which exhibit both peroxidase and catalase activity.

Inactive Fe_2O_3 (Siderac)
Inactive Fe_2O_3 (Baudisch)
} which exhibit only catalase activity.

Sodium pentacyano aquo-ferro salt
Sodium pentacyano aquo-ferri salt
Sodium pentacyano amino-ferri salt
} which exhibit both catalase and peroxidase activity.

In order to obtain comparative results, the following technique was used. The oxides were added to test-tubes in amounts of 100 to 500 mg. and autoclaved. 5 cc. of sterile broth were added later under sterile conditions. As to the ferro-aquo, ferri-aquo, and ferri-amino salts which are soluble and heat-labile, their solutions were filtered through Berkefeld filters and the filtrates added under aseptic conditions to sterile broth, the final concentrations varying from 1/1,000 to 1/1,000,000.

The tubes were inoculated with 8 hour cultures (in plain broth) of the different types of Pneumococcus. The results may be summarized as follows:

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[1] All the compounds were obtained through the courtesy of Dr. Baudisch.

It is apparent that in no case was the plain broth improved by the addition of iron compounds. On the contrary, some of them seem to have a toxic action; such are the Siderac oxides and the ferri-amino salt.

The results were not changed when yeast extract was added to these media.

It is apparent that these iron compounds with or without the addition of yeast extract, do not give to plain broth those growth-promoting properties which are supplied by blood.

TABLE II.

Influence of Iron Compounds on the Growth of Pneumococcus (II R).

Medium	Inoculum (in cc.)					
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Plain broth	+*	+	−*	−	−	−
Blood broth	+	+	+	+	+	+
Plain broth + active Fe_2O_3 (Siderac)	−	−	−	−	−	−
Plain broth + active Fe_2O_3 (Baudisch)	+	+	−	−	−	−
Plain broth + inactive Fe_2O_3 (Siderac)	−	−	−	−	−	−
Plain broth + inactive Fe_2O_3 (Baudisch)	+	+	−	−	−	−
Plain broth + ferro-aquo salt	+	+	−	−	−	−
Plain broth + ferri-aquo salt	+	+	−	−	−	−
Plain broth + ferro-amino salt	+	−	−	−	−	−

* In the presentation of all these results, + or − indicates that growth did or did not develop.

III. Variations of the Growth-Promoting Properties of Plain Broth with Aging of This Medium.

In the course of these investigations, it has been repeatedly observed that when plain broth has been recently autoclaved, it allows the growth of very minute inocula of Pneumococcus. In order to establish this phenomenon more definitely, the following experiment was carried out.

Experiment 3.—Tubes containing 5 cc. of plain broth (1 week old), were autoclaved for 15 minutes, cooled down, and immediately inoculated with varying amounts of pneumococcus culture (D/39/R). Other tubes of the same autoclaved broth were kept for different intervals of time before being inoculated. Table III shows the highest dilution of inoculum with which growth was obtained at the different periods.

Similar results were obtained with other strains of Pneumococcus (Types I, II, III, virulent and avirulent) and with human strains of hemolytic streptococcus. Boiling the broth for 1 hour had the same effect as autoclaving.

With Staphylococcus, growth in "unboiled" broth (3 weeks old) was obtained up to 10^{-5} cc. inoculum, while it took place up to 10^{-8} in recently autoclaved broth.

TABLE III.

Growth of Pneumococcus (D/39/R) in Broth Inoculated at Different Times after Autoclaving.

Medium	Inoculum (in cc.)						
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
"Unautoclaved" broth	+	+	−	−	−	−	−
Broth inoculated immediately after autoclaving	+	+	+	+	+	+	+
Broth inoculated 2 hrs. after autoclaving	+	+	+	+	+	+	+
Broth inoculated 4 hrs. after autoclaving	+	+	+	+	+	−	−
Broth inoculated 8 hrs. after autoclaving	+	+	+	+	−	−	−
Broth inoculated 12 hrs. after autoclaving	+	+	+	−	−	−	−
Broth inoculated 24 hrs. after autoclaving	+	+	−	−	−	−	−
Broth inoculated 48 hrs. after autoclaving	+	+	−	−	−	−	−
Broth inoculated 1 week after autoclaving	+	+	−	−	−	−	−
Broth inoculated 3 weeks after autoclaving	+	−	−	−	−	−	−

It is very important to point out that the effects of boiling or autoclaving are not limited to a short time after the treatment, but extend over 12 hours at least. As has been pointed out elsewhere (12) this indicates that the effect of the treatment is not limited to a mechanical removal of oxygen.

It has already been suggested that the effect of autoclaving or boiling is due to a breaking down or a reduction of oxidized substances

formed by the autoxidation of some constitutents of the broth. If this is the case, similar results should be obtained by chemical methods of reduction. Experiments 4, 5, and 6 are examples of such methods.

Experiment 4.—A lot of broth (2 weeks old) was divided into 2 portions, 1 of which was reduced by hydrogen in the presence of palladinized asbestos, the asbestos being later filtered out in a nitrogen atmosphere. The untreated broth was used as control.

TABLE IV.

Growth of Pneumococcus (D/39/R) in Broth Reduced by Hydrogen.

Medium	Inoculum (in cc.)						
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Plain broth	+	+	−	−	−	−	−
Reduced broth	+	+	+	+	+	+	+

TABLE V.

Influence of Vaseline Seal on the Growth of Different Organisms in Plain Broth.

Organism	Smallest inocula with which growth developed	
	Aerobic cultures	Sealed cultures
Pneumococcus D/39/R	10^{-2}	10^{-4}
A/66/64	10^{-1}	10^{-4}
S. hæmolyticus (L)	10^{-2}	10^{-4}
S/43	10^{-1}	10^{-3}
Staphylococcus aureus	10^{-5}	10^{-8}

The 2 portions were transferred to test-tubes (5 cc. per tube), and their growth-promoting power compared (see Table IV).

It appears that reducing the broth by hydrogen in the presence of palladinized asbestos affects its value in the same manner as boiling or autoclaving.

It has been indicated elsewhere (12) that plain broth kept under vaseline seal develops a reduction potential corresponding to reduced indigo disulfonate. In view of the results of Experiment 4, it was interesting to test whether incubation under vaseline seal would affect the size of the inoculum required to initiate growth.

Experiment 5.—Test-tubes containing 5 cc. of broth (2 weeks old) were inoculated with Pneumococcus, human strains of hemolytic streptococcus, and *Staphylococcus aureus;* some of the tubes were sealed with vaseline immediately after inoculation, while the others were incubated under aerobic conditions. Table V indicates the smallest inocula with which growth developed.

This experiment, the results of which have been repeatedly confirmed, show that initiation of growth is facilitated under vaseline seal. However, the results obtained were not so striking as the ones obtained with "boiled" or "autoclaved" broth, or, as shown later, by the addition of cysteine or ascitic fluid to the broth.

Sterile blood was added to the tubes which had failed to grow, but no growth developed on further incubation, thus indicating that the inoculum was dead. In the case of Pneumococcus, autolysis of the cells may account for the fact, but this explanation does not hold for Streptococcus; the significance of these observations will be discussed later.

IV. Growth of Pneumococcus, Streptococcus hæmolyticus, and Staphylococcus in Cysteine Broth.

Cysteine is an active reducing agent, capable of reducing indigo carmine (13). Its action in facilitating the growth of certain anaerobes has been reported and Quastel and Stephenson (7) suggested that, in the case of *B. sporogenes*, the beneficial action of cysteine was due to the establishment in the medium of a favorable reduction potential.

A preliminary experiment showed that, in the presence of 0.1 per cent cysteine, growth of Pneumococcus (D/39/R), human strains of *S. hæmolyticus* (L), and *Staphylococcus aureus* could be obtained with inocula of 10^{-7}, 10^{-7}, and 10^{-9} cc. of broth cultures, respectively.

Experiment 6.—Plain broth was treated with different concentrations of cysteine (0.002 per cent, 0.005 per cent, 0.01 per cent, 0.02 per cent, 0.03 per cent, 0.1 per cent), in order to determine the smallest concentration at which that substance would be active. The tubes were inoculated 12 hours after treatment and incubated under aerobic conditions for 72 hours. The organisms used were those described in the experimental methods.

This experiment shows that cysteine was still active in as high a dilution as 0.005 per cent. With very small inocula (10^{-6}, 10^{-7}, 10^{-8}) growth developed only after 2 to 3 days, this indicating that cysteine was still active at the time.

TABLE VI.

Smallest Inocula (in cc.) with Which Growth Developed in Presence of Different Concentrations of Cysteine.

Organism	Concentration of cysteine						
	0.1	0.03	0.02	0.01	0.005	0.002	0
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	
Pneumococcus 1/193/R	10^{-7}	10^{-8}	10^{-8}	10^{-8}	10^{-7}	10^{-4}	10^{-2}
1/200/5	10^{-8}	10^{-7}	10^{-7}	10^{-6}	10^{-6}	10^{-3}	10^{-2}
D/39/R	10^{-8}	10^{-8}	10^{-8}	10^{-6}	10^{-8}	10^{-5}	10^{-3}
D/39/45	10^{-7}	10^{-7}	10^{-8}	10^{-7}	10^{-8}	10^{-3}	10^{-2}
M/3/R	10^{-8}	10^{-8}	10^{-8}	10^{-6}	10^{-7}	10^{-4}	10^{-2}
A/66/64	10^{-6}	10^{-8}	10^{-7}	10^{-6}	10^{-6}	10^{-3}	10^{-2}
S. hæmolyticus S/23/G	10^{-8}	10^{-7}	10^{-7}	10^{-6}	10^{-6}	10^{-3}	10^{-2}
S/23/76	10^{-7}	10^{-7}	10^{-7}	10^{-7}	10^{-6}	10^{-4}	10^{-2}
Staphylococcus aureus	10^{-9}	10^{-9}	10^{-8}	10^{-8}	10^{-8}	10^{-7}	10^{-5}

TABLE VII.

Effect of Fumaric and Succinic Acids (0.2 Per Cent in Weight) on the Growth of D/39/R.

Medium	Inoculum (in cc.)			
	10^{-1}	10^{-2}	10^{-3}	10^{-4}
Plain broth	+	+	−	−
Plain broth + succinic acid	+	+	−	−
Plain broth + fumaric acid	+	+	−	−

TABLE VIII.

Smallest Inocula Required for Growth to Develop in Plain Broth and Ascitic Fluid Broth.

Organism	Plain broth	Ascitic fluid broth	
		Heated	Unheated
Pneumococcus (D/39/R)	10^{-1}	10^{-2}	10^{-7}
S. hæmolyticus (L)	10^{-1}	10^{-2}	10^{-6}
Staphylococcus aureus	10^{-4}	?	10^{-9}

It is interesting to test the influence of other reducing organic substances. Succinic acid is such a substance, its oxidized form being fumaric acid.

Experiment 7.—The effect of the addition of plain broth (2 weeks old) of fumaric and succinic acids on the aerobic growth of Pneumococcus is recorded in Table VII.

As far as the experiment shows, the addition of succinic and fumaric acids does not affect the growth of Pneumococcus (D/39/R). The only difference observed was that growth developed in 24 hours in the succinic acid tubes inoculated with 10^{-2} cc., while it took 48 hours in the plain broth and fumaric acid broth with the same inoculum.

Experiment 8.—Ascitic fluid broth (5 per cent ascitic fluid, added to 3 weeks old plain broth) was tested for its value in the initiation of growth of small inocula of facultative anaerobes. In some of the tubes the ascitic fluid had been boiled before addition to the broth. A typical protocol is presented in Table VIII.

These results have been confirmed with many other strains of the same bacterial species. They show that unheated ascitic fluid broth allows the growth of very small inocula of the organisms used. Ascitic fluid seems to be inactivated by boiling.

DISCUSSION.

It is apparent that the growth-promoting properties of plain broth do not remain constant. Fresh broth used within a few hours after its preparation, usually grows Pneumococcus, human strains of hemolytic streptococcus, and *Staphylococcus aureus*, even when one or a very few cells are used as inoculum (10^{-7}, 10^{-9} cc. of plain broth cultures). Within 24 hours, the broth has so changed that it can grow the same organisms only if large inocula are used (10^{-2}, 10^{-3} cc.) and after 3 weeks, the broth has become very poor. However, this same broth can be restored to its original value by autoclaving, boiling, or reducing with hydrogen in the presence of palladinized asbestos, as well as by the addition of small amounts of reduced cysteine, but not by the addition of succinic acid. The addition of ascitic fluid and of very small amounts of blood also serves to restore its value.

It is probably fallacious to attempt to explain the results obtained with all these procedures from one single point of view. However, it may be worth while to point out some possible correlations.

Let us first consider the "deterioration" of plain broth on aging. The fact that this "deterioration" can be completely or partially corrected by reducing the medium with hydrogen and with cysteine, or by keeping it under vaseline seal (condition under which the broth is known to develop highly reducing potentials (12)), suggests strongly that this "deterioration" is connected with some oxidation or oxygenation process. The influence of autoclaving and boiling may lead to the same conclusion.

It is hardly probable that the results can be explained by a purely mechanical removal of the oxygen. Studies with indicators of oxidation-reduction potentials have suggested that boiling or autoclaving has more than this purely mechanical effect (12). It has been shown that recently boiled or autoclaved broth maintains the indophenols in a reduced condition for several hours, even under conditions of active aeration. This indicates that the oxidation potential of the broth is lowered either by the reduction of some reversible system of oxidation-reduction, or by the breaking down of a highly oxidized system. A similar conclusion can be reached from a consideration of the results of Experiment 3, in which it is shown that the influence of boiling lasts for over 12 hours—even if the broth is thoroughly aerated.

Blood is an active reducing agent and is also known to possess catalytic properties. Whether the high dilution at which it is still active justifies the assumption that its action is due to a quantitative reduction is problematical. It is possible that the action of blood results in a catalytic breaking down of the peroxides that appear to form in aerated plain broth. In such a case, the inactivity of the iron catalysts used in Experiment 2 would have to be traced to a lack of specific affinity between the iron compounds and the "organic peroxide" of the broth. Finally, it must be remembered that blood introduces formed elements in the medium. It is certain that a bacterial cell adsorbed by, or simply in the vicinity of, a blood corpuscle, finds local conditions markedly different from those prevailing in the rest of the medium. That such conditions are highly reducing is probable.

Little is known of the properties of ascitic fluid. Confirming others

(14), we have found that its reducing capacity is very small, but the factor intensity should be measured by electrometric methods. On the other hand the sample of ascitic fluid used in these experiments gave the peroxidase and catalase tests; it is possible that these catalytic properties were due to the presence of occult blood in the fluid. In such a case, the action of ascitic fluid would be only another example of the activity of small concentrations of blood.

Concerning the reducing intensity of succinic acid, Thunberg (15) reported that equimolecular mixtures of succinic and fumaric acids kept under anaerobic conditions with methylene blue (in the presence of succinodehydrogenase) give only a partial reduction of the dye. This indicates that the system has a very low reduction intensity, much inferior to that of cysteine (which can reduce indigo carmine) and may account for its inactivity. Furthermore, it is probable that questions of specific affinity have to be considered here.

Any problem of growth is of course a problem of reaction between the organism and the environment. So far, our discussion has been concerned only with the environment. Let us now consider the cell as affected by these environmental conditions. We know that the pneumococcus cell is extremely sensitive to changes in the oxygen tension, its thermostable autoxidizable substance becoming alternately oxidized or reduced according to whether the conditions are aerobic or anaerobic, and the thermolabile cellular constituent becoming irreversibly inactivated by slow oxidation. It is to be expected, therefore, that the condition of the cell will be greatly affected by the oxidation potential of the medium. It is also probable that the condition of oxidation or reduction of the cell is not without effect on its ability to multiply.

Our results seem to be best explained by the following hypothesis.

Oxidation processes bear a definite relation to the size of inoculum required to initiate the growth of Pneumococcus, Streptococcus, and Staphylococcus. As we prepare it, plain broth contains reducing autoxidizable substances. In contact with air these substances give rise either to the oxidized form of a reversible oxidation-reduction system, or, by irreversible oxidation, to highly oxidized substances (of the nature of so called "peroxides"). The organisms studied in this work require a medium with a definite range of oxidation-reduction potential for cell multiplication to occur (7, 9, 16). "Oxidized"

broth has a potential outside this range. Such a condition may be corrected in different ways.

(*a*) The bacterial cell itself is equipped with an active reducing system. When a large inoculum is used, enough reducing substances are introduced (from the bacterial cells and the reduced medium carried with them) to reduce the "oxidized" broth and bring it back to favorable conditions.

(*b*) Addition of cysteine and blood, and reduction by means of hydrogen, serve the same purpose. Blood may be active also by catalyzing the breaking down of the hypothetical "peroxides."

(*c*) It is more difficult to see how boiling or autoclaving may bring about the reduction of a reversible system; it is more probable that they cause a breaking down of the oxidized substance.

(*d*) When incubated under vaseline seal, plain broth develops a high reduction potential (12). It may seem surprising that, under such conditions, the results are not so favorable as those obtained by reducing the broth in some other way. A possible explanation is that the reduction of the "oxidized" substances of the broth under vaseline seal is only a slow process; before they are all reduced by the broth itself, they exert some toxic action on the bacterial cells and a part of the cells of the inoculum are killed (Experiment 5).[2]

SUMMARY.

The growth of many pathogenic organisms in plain meat infusion broth is possible only when a large inoculum is used.

This requirement is much less strict when the broth cultures are

[2] It is realized that the oxidation-reduction potential of the broth is only one of many factors to be considered in the problem of initiation of growth. In the course of this work, several batches of plain broth prepared according to the standard method, have been found to be very poor for the growth of Pneumococcus and their value could not be enhanced by any of the treatments enumerated above.

It has been observed that samples of broth, several months old, become completely inadequate for the growth of Pneumococcus after they have been boiled, but that their value can be restored by addition of cysteine. This effect of boiling on very old broth is not as yet understood.

Recent experiments have shown that cystine could be replaced by equivalent concentrations of thioglycollic acid.

incubated (*a*) under anaerobic conditions, (*b*) in fresh media very recently boiled or autoclaved, (*c*) in fresh media reduced by means of hydrogen, or to which small amounts of cysteine or of blood have been added.

It is suggested that these findings can be accounted for by assuming that the bacterial species under consideration can multiply only in media the oxidation potential of which is below a critical value.

The favorable growth conditions obtained by the procedures enumerated above may be attributed to the establishment of a proper reduction potential in the medium; the same result is obtained by using a large inoculum, owing to the reducing properties of bacterial cells.

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