

STUDIES ON CARBON DIOXIDE

IV. THE INFLUENCE OF GASEOUS ENVIRONMENT ON GROWTH AND TOXIN PRODUCTION OF *C. DIPHTHERIAE*¹

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INTRODUCTION

Since Roux and Yersin discovered the presence of a specific soluble toxin in the culture filtrates of *C. diphtheriae* considerable effort has been made to determine the factors underlying the formation of the toxin. This problem is not only of pronounced theoretical interest, but also has a very important practical bearing on the commercial production of potent toxin and anti-toxin.

Upon reviewing the available literature concerned with the production of diphtheria toxin, one finds little mention of the influence of gaseous environment on growth and toxin formation, aside from the statement that a rich oxygen supply is highly essential. Roux and Yersin (1890 and 1894), Aronson (1894), Spronck (1895), Park and Williams (1896), Madsen (1897) and Martin (1898) at times observed more rapid and abundant toxin formation in cultures aerated with ordinary air than in those which were incubated under the usual conditions. However, passing a current of air over the cultures also resulted in marked irregularity in the toxicity of the filtrates and caused rapid destruction of the toxin once it was formed. Consequently, aeration of diphtheria cultures with ordinary air was considered more deleterious than beneficial.

That carbon dioxide apparently has no inhibiting effect on

¹ This paper embodies part of the work presented in the Senior author's Doctorate Thesis deposited in the Yale University Library.

Loeffler serum cultures of *C. diphtheriae* was shown by Fränkel (1889), who observed that growth was as luxuriant under greatly increased carbon dioxide as in the ordinary air. More recently Lorentz (1923) reported a higher percentage of positive findings by incubating field cultures from suspected diphtheritic patients under added carbon dioxide than was obtained by the usual method.

The favorable influence of carbon dioxide on a certain limited number of bacterial species has been known for some time. The meningococcus was found to grow more luxuriantly in an atmosphere containing from 10 to 20 per cent carbon dioxide than under ordinary environmental conditions, by Cohen and Fleming (1918), Gates (1919) and Phelon, Duthie and M'Leod (1927). Similar observations have been made on the gonococcus by Chapin (1918), and on *Bact. abortus* by Huddleson (1921), and Smith (1924). There has been considerable speculation as to how the favorable influence is exerted; however, but little significance was attached to the carbon dioxide per se. Sierkowski and Zajdel (1924) suggested that carbon dioxide may play a rôle as hydrogen ion regulator in all bacterial cultures. The extensive researches of Valley and Rettger (1926 and 1927) have shown that all bacteria thus far studied by them, including *C. diphtheriae*, are unable to grow in the complete absence of carbon dioxide as such.

These observations, together with the fact that the diphtheria organism is exposed to a more or less carbon dioxide-rich gaseous environment in the nose and throat, suggested that carbon dioxide may play an important rôle in the growth and toxin production of *C. diphtheriae*.

The present investigation is concerned chiefly with the influence of different gas mixtures of nitrogen, oxygen and carbon dioxide on the growth and toxin production of *C. diphtheriae*, with a view to acquiring more complete information regarding the principles governing uniform toxin production.

METHODS

Cultures. The well known Park-Williams No. 8 strain of *C. diphtheriae* was obtained from the Hygienic Laboratory and

maintained on Loeffler's serum according to the method recommended by Gibbs and Rettger (1927). Inoculation of the culture flasks, unless otherwise stated, was accomplished by floating a 4 mm. loopful of the surface growth of an "acclimated" meat-infusion-peptone broth culture on the surface of the medium. This method was found to result in more rapid growth and toxin production than was obtained by adding 1 cc. of broth culture to each flask in the usual manner.

Medium employed for toxin production. The commonly used medium containing beef infusion, 2 per cent Difco-Proteose Peptone and 0.5 per cent NaCl was employed. The final reaction was adjusted to pH 7.5. After clarification by heat, the filtered medium was distributed in 500 cc. Erlenmeyer flasks, 90 cc. to each flask. Final sterilization was accomplished by autoclaving at fifteen pounds extra pressure for fifteen minutes.

The following determinations were made on each test culture after definite periods of incubation:

Hydrogen ion concentration, by the colorimetric method of Clark and Lubs (1917).

Amino-nitrogen, by the Sørensen Formol Titration Method as employed by Slanetz and Rettger (1928). The method is essentially that described by Brown (1923), under "Method B."

Ammonia-nitrogen, by the Van Slyke and Cullen modification of the Folin air current method, as used by Slanetz and Rettger (1928). No attempt was made to distinguish between free ammonia and volatile amines, all volatile alkaline substances occurring in a given test culture being calculated as free ammonia.

Bacterial sediment per 100 cc. of culture, by centrifuging an aliquot portion of culture in a calibrated Hopkins centrifuge tube.

Number of viable organisms per cubic centimeter of culture, by the plating method. Meat-infusion-peptone-glucose agar, the reaction of which had been adjusted to pH 7.5 just before using by the addition of sterile normal NaOH solution, was found to give fairly consistent plate counts. The plates were incubated under an atmosphere containing from 3 to 5 per cent carbon dioxide and 20 per cent oxygen for a period of four days. The colonies were then counted with the aid of a hand lens. Plates

incubated under these conditions yielded higher counts and larger colonies, as a rule, than plates incubated in the usual way.

Preservation of culture filtrates. After carrying out the previously mentioned tests on each culture, 0.5 per cent phenol was added as a preservative. The phenol-treated culture was allowed to stand over night in the ice-chest. On the following morning the supernatant fluid was separated from the sediment by decantation and stored in the ice-chest in tightly stoppered containers.

Toxicity of culture filtrates. a. Intracutaneous method. The intracutaneous test, originated by Römer and Sames (1909) and later developed and applied by Glenny and Allen (1921), was used in determining the toxicity of experimental toxins. Results were recorded in Ln/500 doses. One Ln/500 dose, so far as experiments reported herein are concerned, may be defined as the smallest amount of toxin which when injected intracutaneously, together with $\frac{1}{100}$ unit of antitoxin,² caused a small area of necrosis distinguishable five days after the injection.

Briefly, the procedure employed is as follows: A portion of the test toxin was diluted with sterile physiological salt solution so that 0.05 cc. of the resulting solution contained the exact amount of toxin to be injected. To 1 cc. of the diluted toxin contained in a sterile test tube was added 1 cc. of a solution which contained $\frac{1}{100}$ units of antitoxin.² One-tenth cubic centimeter of the resulting toxin-antitoxin mixture then contained the desired amount of toxin for injection, and also $\frac{1}{100}$ unit of antitoxin. After standing fifteen minutes at room temperature, exactly 0.1 cc. of the toxin-antitoxin mixture was drawn into a tuberculin syringe equipped with a fine gauge needle and injected into the shaven skin of a white guinea pig, as near the surface as possible. As many as six injections were made at one time on a large pig, allowing a space of at least one inch between the injections. Usually 3 injections were made on each side of the median ventral line.

b. Subcutaneous method (L+dose). The procedure described

² One unit of antitoxin in this case is defined as the amount of antitoxin which will exactly neutralize one minimal lethal dose of diphtheria toxin.

in Hygienic Laboratory Bulletin No. 21 (April, 1905), was used in the determination of the L+ dose of toxin.

EXPERIMENTAL

The reliability of the intracutaneous method of standardizing diphtheria toxin

In undertaking an investigation which requires toxicity determinations on large numbers of experimental toxins, it is necessary to select a method which is economical but at the same time fairly accurate.

The flocculation test described by Ramon (1922) is economical and can be carried out rapidly. However, while Ramon's method may measure the antigenic properties of a mixture of toxin and toxoid, it does not necessarily give results which indicate the toxicity of a toxin solution, and for that reason the test was not employed in this investigation.

Of the various methods which depend upon animal inoculation the intracutaneous test appears to be the most economical and is most rapidly carried out. As to its reliability, Glenny and Allen (1921) and Watson and Wallace (1923) report that the results obtained by the intracutaneous method correlate fairly well with those obtained by the subcutaneous test, although no exact relation appeared to exist between the two. The intracutaneous method has been used by Hartley (1922) and by Watson and Wallace (1924) in determining the toxicity of experimental toxins.

Before adopting the intradermal method in the routine testing of culture filtrates, several tests were made to determine its reliability, and to select what may be termed an "end-point." A definite amount of toxin will produce an area of redness 1 cm. in diameter which disappears shortly after forty-eight hours. A slightly larger amount will produce an area of redness 2 cm. in diameter, followed on the third or fourth day by oedema and slight necrosis at the site of the injection; while a still larger amount will cause a distinct area of necrosis 0.5 to 2.0 cm. in diameter in three to five days. This being the case, it is necessary to decide

upon the character of the reaction which will be considered positive.

TABLE 1

Showing the effect of injecting different amounts of toxin intradermally, together with 1/500 unit of antitoxin

AMOUNTS OF TOXIN INJECTED	REACTION PRODUCED				
	24 hours	48 hours	72 hours	4 days	5 days
<i>cc.</i>					
0.0003	Redness 1 cm.	0	0	0	0
0.0004	Redness 1 cm.	Redness 1 cm.	0	0	0
0.0005	Redness 1.5 cm.	Redness 1.5 cm.	Slight edema	Peeling 1 cm.	Peeling 1 cm.
0.00075*	Redness 1.5 cm.	Redness 1.5 cm.	Edema 1 cm.	Necrosis 0.3 cm.	Necrosis 0.5 cm.
0.001	Redness 2 cm.	Edema 2 cm.	Necrosis 0.3 cm.	Necrosis 1 cm.	Necrosis 1 cm.
0.0015	Redness 2 cm.	Edema 2 cm.	Necrosis 1 cm.	Necrosis 2 cm.	Necrosis 2 cm.

* Represents the Ln/500 dose. Peeling means sloughing at the site of the injecting.

TABLE 2

Showing the relative susceptibility of guinea pigs to intracutaneous injection of diphtheria toxin

TOXINS	AMOUNT INJECTED	REACTION OBSERVED ON THE FIFTH DAY	
		Guinea pig a	Guinea pig b
	<i>cc.</i>		
IV-20-9	0.0010	1.0 cm. of necrosis	1.0 cm. of necrosis
IV-10-5	0.00075	0.6 cm. of necrosis	0.5 cm. of necrosis
IV-D-9	0.0015	0.8 cm. of necrosis	0.5 cm. of necrosis
S-C-17	0.0004	1.0 cm. of necrosis	0.5 cm. of necrosis
R-C	0.0005	1.0 cm. of necrosis	0.8 cm. of necrosis
R-S-D	0.0010	0.5 cm. of necrosis	0.5 cm. of necrosis
XIV-D-20	0.0005	0.5 cm. of necrosis	0.6 cm. of necrosis

Table 1 illustrates the effect produced by injecting varying amounts of a given toxin, together with $\tau\tau$ unit of antitoxin, intracutaneously.

The smallest amount of toxin which, when injected intracutaneously with $\frac{1}{100}$ unit of antitoxin, causes an area of necrosis 0.5 cm. in diameter, distinguishable five days after the injection, was selected as the end-point. Accordingly one Ln/500 dose of toxin was present in 0.00075 cc. of the above toxin.

In order to determine the relative susceptibility of guinea pigs to intracutaneous injections of toxin, similar amounts of toxin-antitoxin mixtures were injected into the skin of different guinea pigs. A few of the results obtained are recorded in table 2.

TABLE 3
Showing the relation of the Ln/500 dose to the L+ dose

TOXINS	Ln/500 dose	L+ dose	$\frac{\text{Ln/500}}{\text{L+}}$
	cc.	cc.	
X-5-5	0.0002	0.16	1-800
X-5-10	0.0002	0.15	1-750
X-30-10	0.0002	0.15	1-750
X-50-10	0.0002	0.14	1-700
XIII-15-10	0.00015	0.15	1-1000
A-8	0.0010	0.60	1-600
Hyg.	0.0003	0.20	1-667
IV-10-5	0.0007	0.42	1-600
XIV-5-20	0.0002	0.14	1-700

$$\text{Average } \frac{\text{Ln/500}}{\text{L+}} = 1/700.$$

The results obtained in the two animals checked fairly closely, on the whole. However, a slight variation in the reaction produced by injecting similar doses of a given toxin did occur in a few instances; this was possibly due either to a slight difference in the susceptibility of the guinea pigs injected, or to leakage at the site of the injection, or to a difference in the depth of the injection. It was found necessary, of course, to make all injections as near the surface of the skin as possible, in order to obtain the characteristic local necrosis, instead of the diffuse type which is more apt to follow the deeper injections.

The toxicity of a number of culture filtrates was determined by the intracutaneous and subcutaneous methods for the purpose of

ascertaining the relation of the Ln/500 dose to the L+ dose. The results are recorded in table 3.

These results show that, while no fixed relation exists between the Ln/500 dose and the L+ dose, the latter is approximately 700 times the former. A certain amount of disagreement between the two tests is to be expected: this may be due to several causes, namely, the nature of the toxin injected, and a difference in the susceptibility of the guinea pigs to both the skin test dose and the L+ dose. That guinea pigs vary in their susceptibility to L+ dose toxins has been shown, among others, by Craw and Dean (1907,) and Südmerson and Glenny (1909).

In view of these observations, the authors feel that the intracutaneous test, as described, may be considered a reliable means of approximating the toxicity of experimental toxins. While the skin test is probably not as accurate as the subcutaneous test (determination of the L+ dose), its advantages with respect to saving in animals, materials and time far outweigh its disadvantages.

DESCRIPTION OF AERATION APPARATUS EMPLOYED IN THIS INVESTIGATION

In order to study the effect of definite gas mixtures on growth and toxin production of *C. diphtheriae*, it was necessary to devise an apparatus whereby cultures could be grown in the different gaseous environments. The use of a sealed container would obviously be unsatisfactory for this purpose, as the oxygen tension would be reduced and the carbon dioxide increased during the growth of the culture, and, hence, no definite equilibrium maintained. Consequently, it seemed prudent to aerate the test cultures with known gas mixtures at such a rate that the gas within the culture flasks would remain uniform as to composition. A diagram of the aeration apparatus used in the succeeding experiments is given in figure 1. Arrows indicate the course of the gas mixture.

Three 500-cc. Erlenmeyer flasks equipped with ordinary cotton plugs and containing 90 cc. of sterile broth were inoculated in the usual way. A special cotton plug bearing two bent glass

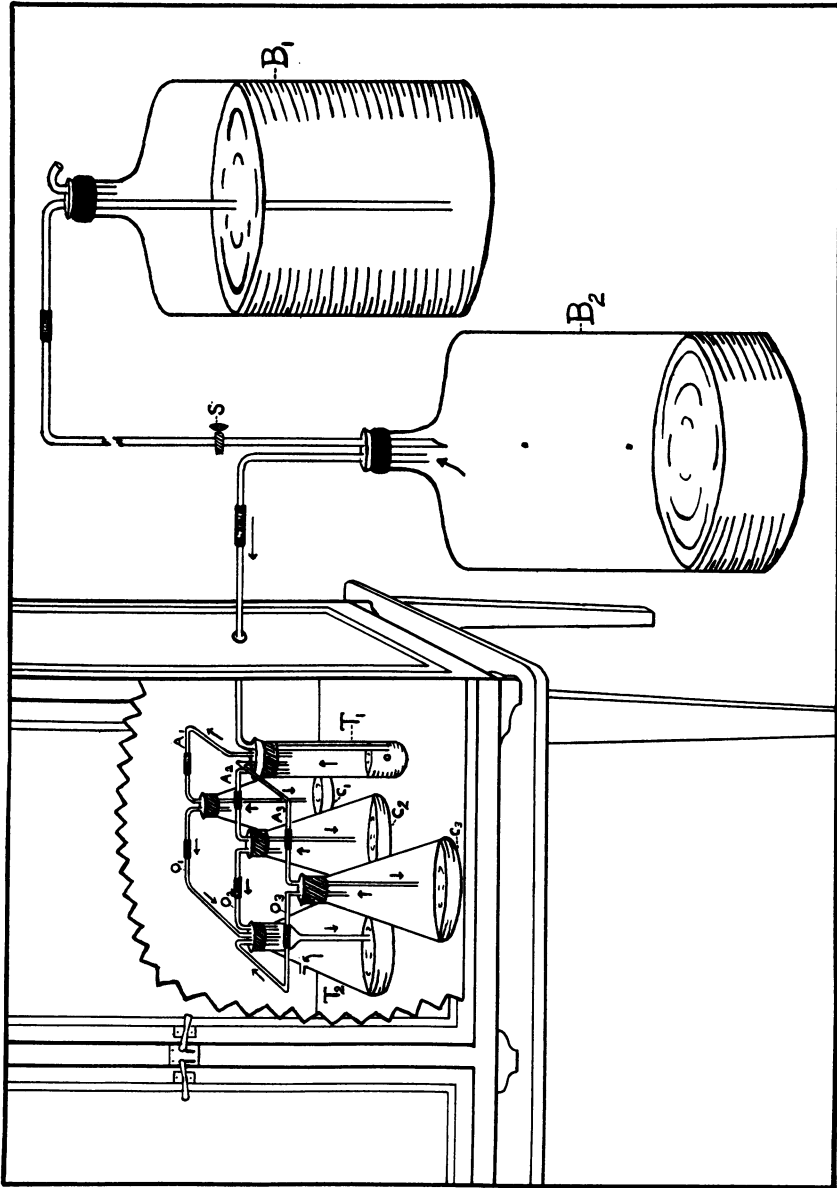


Fig. 1

tubes (inlet and outlet tubes) which had been previously wrapped in paper and sterilized in the oven, was then substituted for the ordinary cotton plug in each of the three flasks. The special plugs were pushed down the neck of the culture flasks a distance of 0.5 cm. below the mouth. The flasks were then made airtight, except for the inlet and outlet tubes, by placing a layer of paraffin at least 5 mm. in depth over the special plug. The 3 flasks (C_1 , C_2 and C_3) to be aerated were then placed in the system as shown in the accompanying diagram.

A 12-liter bottle (B_1) filled with water or $N/100$ sulphuric acid, depending on whether or not the test atmosphere contained carbon dioxide, and placed on a shelf at a height of 4 feet over B_2 . B_1 , was connected with a second 12-liter bottle (B_2) which contained the test gas mixture, by means of suitable glass tubing and a one-way stopcock (S).

The stopcock (S) was then opened and a siphon started between B_1 and B_2 by applying air pressure inside B_1 . The rate of flow was regulated by means of stopcock (S) which indirectly governed the rate of gas flow inside the culture flasks. As shown in figure 1, a stream of the test atmosphere flowed from B_2 to a trap (T_1) where it was equally divided between inlet tubes (A_1 , A_2 and A_3). In this way separate streams of test gas mixture passed through the culture flasks (C_1 , C_2 and C_3). Upon leaving the culture flasks through outlet tubes (O_1 , O_2 and O_3) the gas mixture passed through a second trap (T_2) and out into the open air.

The traps (T_1 and T_2) contained 10 per cent sodium hydroxide in instances where the test atmospheres were CO_2 -free; at other times they contained 5 per cent sulphuric acid. T_1 served several purposes: (1) it saturated the test atmosphere with moisture, thereby preventing loss of water from the culture; (2) it removed bacteria from the applied gas mixture; (3) it maintained an equal pressure on each pipe line leading to the culture flasks, thereby assuring an even distribution of gas to each culture; and (4) when supplied with 10 per cent sodium hydroxide, it removed traces of carbon dioxide from the test atmospheres. T_2 prevented an inward diffusion of gases from the outside air.

Three aeration systems similar to the one described were set

up in one incubator (large Thelco) and employed throughout the present investigation. The plan and results of the different experiments are presented in the following pages.

Aeration of cultures with carbon dioxide-free air,³ ordinary air, and air containing 3 per cent CO₂⁴

Sixteen flasks containing 90 cc. of the same lot of meat-infusion-peptone broth were inoculated, each with 1 cc. of a twenty-four-hour culture of *C. diphtheriae*, and placed in the 37°C. incubator. Four of the flasks were aerated with CO₂-free air, four with ordinary air, and four with air containing 3 per cent carbon dioxide; the fourth set of flasks was incubated under ordinary atmospheric conditions. Approximately 100 cc. of test gas mixture were passed over each aerated culture per hour. The results are recorded in table 4.

Toxicity (chart 1). Cultures aerated with air containing 3 per cent carbon dioxide were characterized by greater and more rapid toxin formation than were the cultures incubated under ordinary atmospheric conditions. Maximum toxicity was attained on the fifth day and was maintained at a relatively constant level during the remainder of the twenty-day incubation period. On the other hand, cultures aerated with either CO₂-free air or ordinary air were less toxic than the control cultures and showed a marked irregularity in toxin content. A comparison of chart 4 with chart 1 shows that appreciable amounts of toxin did not appear in any of the cultures until after the period of maximum growth.

Hydrogen ion concentration (chart 2). The hydrogen ion concentration remained within the optimum range for toxin formation (between pH 7.5 and pH 8.0) in the cultures aerated with

³ Valley and Rettger (1927) have shown that complete removal of carbon dioxide from the culture medium and from the atmosphere in contact with the medium prevents the growth of *C. diphtheriae*. In this experiment no attempt was made to remove carbon dioxide from the culture medium. As growth was obtained in the cultures aerated with CO₂-free air, it must be assumed that sufficient CO₂ was dissolved in the medium to initiate growth.

⁴ The results reported in this experiment have been presented in a preliminary report by Plastring and Rettger (1927).

air containing 3 per cent carbon dioxide. On the other hand, the pH values of the cultures aerated with ordinary air and CO₂-free air increased from pH 7.5 to pH 9.0 by the fifth day of incubation and remained at pH 9.0 throughout the remainder of the incubation period. The increase in pH of the control cultures followed

TABLE 4

Showing the results of aeration with CO₂-free air, ordinary air, and air containing 3 per cent CO₂, as compared with those obtained under ordinary conditions

CULTURE	DAYS INCUBATED 37°C.	Ln/500 DOSE	pH	NH ₃ -N in 100 cc.	SEDIMENT IN 100 cc.	GROWTH	VIABLE CELLS PER CUBIC CENTIMETER
		cc.		cc.	cc.		
Controls incubated under ordinary conditions	3	0.01	8.6	27.8	1.1	M. C.	250,000,000
	5	0.003	8.6	26.2	1.2	M. C.	325,000
	8	0.0018	8.9	23.3	1.1	H. C.	2,200
	20	0.005	8.9	13.4	0.8	M. C.	60,000
Cultures aerated with CO ₂ -free air	3	0.01	8.4	28.7	1.2	H. C.	360,000,000
	5	0.0025	8.8	29.0	1.2	H. C.	257,000
	8	0.0025	9.0	28.0	1.0	Br. C.	2,200
	20	0.005	9.0	27.0	1.0	Br. C.	1,000
Cultures aerated with ordinary air	3	0.01	8.4	27.7	1.2	H. C.	63,000,000
	5	0.01	9.0	29.1	1.2	H. C.	26,000
	8	0.004	9.0	28.0	1.1	Br. C.	3,000
	20	0.008	9.0	26.0	1.1	Br. C.	8,000
Cultures aerated with air containing 3 per cent CO ₂	3	0.01	7.8	29.0	1.2	H. C.	315,000,000
	5	0.0008	7.8	30.7	1.2	H. C.	227,000,000
	8	0.0008	8.0	31.1	1.2	H. C.	95,000,000
	20	0.001	8.0	31.7	1.2	Br. C.	13,000,000
Medium			7.5	7.0			

M. C. = moderate crust; H. C. = heavy crust; Br. C. = broken and partly sunk crust.

somewhat the same general course, except that alkalization was more gradual, the maximum being reached on the eighth day.

Ammonia production (chart 3). The ammonia content of all cultures showed a sharp increase during the first three days of

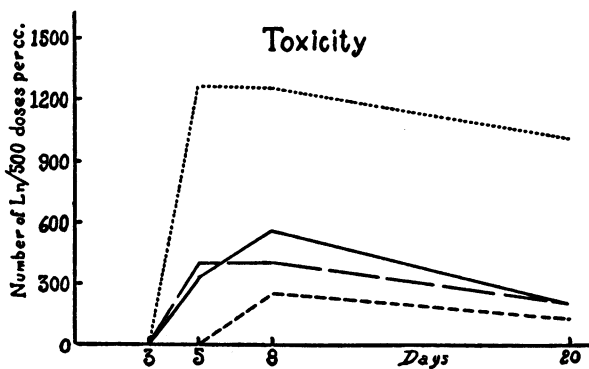


CHART 1

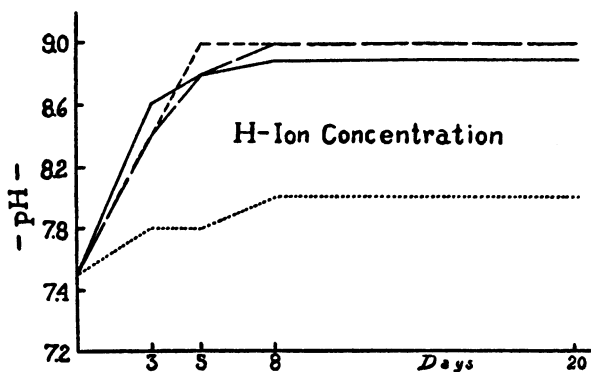


CHART 2

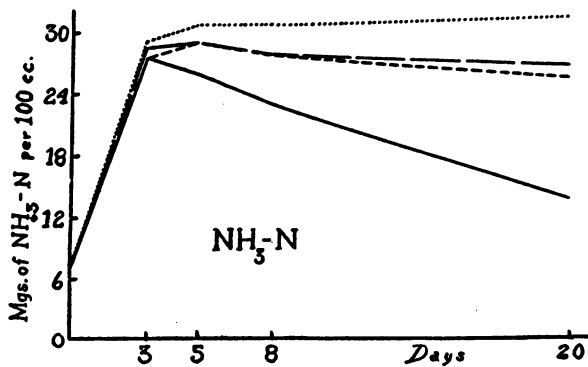


CHART 3

— control.
 - - - aerated with ordinary air.

incubation. In the control cultures and in those aerated with CO_2 -free air or ordinary air this increase was followed by a gradual decline, while a continuous increase occurred in the flasks aerated with air containing 3 per cent carbon dioxide. Apparently the ammonia content of cultures grown under an increased carbon dioxide tension is an indication of the pronounced peptolytic activity of the diphtheria organism.

Plate count (chart 4). The number of viable organisms per cc. in all cultures reached a maximum during the first three days of incubation. In the cultures aerated with an atmosphere containing 3 per cent carbon dioxide the plate count remained at a

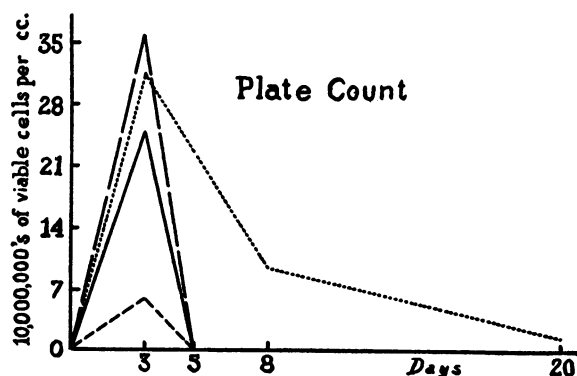


CHART 4

----- aerated with CO_2 -free air.

..... aerated with air + 3 per cent CO_2 .

comparatively high figure during the entire twenty-day incubation period. On the other hand, a sharp drop occurred immediately after the maximum count was reached in the control cultures, and in the cultures aerated with either CO_2 -free air or ordinary air; this is presumably due to the marked increase in alkalinity of the culture medium during the period of maximum growth.

Bacterial sediment. The amount of bacterial sediment produced in the cultures aerated with air containing 3 per cent carbon dioxide surpassed that of the controls. A maximum was reached during the first three days of growth, which remained unchanged

during the remainder of the twenty-day incubation period. Aeration of the cultures with CO₂-free air or ordinary air, under the conditions of the experiment, also resulted in increased rate of growth during the first three days of incubation.

Summary. The results presented here show that carbon dioxide plays an important rôle in the accumulation of toxin in cultures of *C. diphtheriae*. Under an atmosphere of air containing 3 per cent carbon dioxide toxin formation was greatly accelerated and the total amount of toxin accumulating in a given culture was much greater than in a culture grown under the usual conditions, or aerated with either CO₂-free air or ordinary air. Furthermore, an increased carbon dioxide tension over the culture medium apparently prevented, to a large extent, destruction of the toxin once it was formed.

Aeration of broth cultures of *C. diphtheriae* with an atmosphere containing 3 per cent carbon dioxide also favored abundant growth and prevented the usual rapid destruction of the bacterial cells after the period of maximum growth.

Aeration of cultures with atmospheres containing different concentrations of carbon dioxide

In order to determine the carbon dioxide tension most conducive to maximum growth and toxin accumulation, broth cultures of *C. diphtheriae* were aerated with atmospheres containing different percentages of carbon dioxide and approximately 18.5 per cent oxygen.

Twelve flasks of peptone-meat-infusion bouillon were prepared, using Difco-Proteose Peptone, and inoculated with 1 cc. of broth culture of *C. diphtheriae*. These culture flasks were divided into 4 groups, 3 flasks in each group. Group I was incubated under ordinary atmospheric conditions and served as the control, while Groups II, III and IV were aerated with atmospheres containing 5, 10 and 20 per cent carbon dioxide, respectively. One flask was removed from each group after five, nine and twenty days incubation, and the usual determinations made on each. The results are recorded in table 5.

Toxicity (chart 5). The toxicity of cultures grown under a gas mixture containing 10 per cent carbon dioxide and 18.5 per cent oxygen was somewhat greater than that of those grown under atmospheres containing either 5 or 20 per cent carbon dioxide. However, all cultures grown under an increased carbon dioxide tension were more toxic than the controls. No significant deterioration of toxin occurred in the cultures which were incu-

TABLE 5
Showing the influence of different amounts of carbon dioxide

CULTURE	NUM- BER OF DAYS INCUBATION	Ln/500 DOSE	pH	AMINO- N PER 100 CC.	NH ₃ -N PER 100 CC.	SEDI- MENT IN 100 CC.	CHARAC- TER OF GROWTH	VIALE CELLS PER CUBIC CENTIMETER
		cc.		mgm.	mgm.	cc.		
Controls incubated under ordinary conditions	5	0.0017	9.1	73.0	16.5	0.95	M. C.	536,000
	9	0.0015	9.1	79.4	14.2	1.00	M. C.	56,000
	20	0.0025	9.0	77.1	8.0	0.95	M. C.	0
Aerated with air con- taining 5 per cent CO ₂	5	0.001	8.0	79.5	24.8	1.2	H. C.	51,000,000
	9	0.001	8.1	80.4	28.4	1.2	H. C.	40,000,000
	20	0.001	8.2	74.1	34.1	1.2	H. C.	4,800,000
Aerated with air con- taining 10 per cent CO ₂	5	0.0007	7.9	76.9	25.3	1.0	H. C.	65,000,000
	9	0.0008	7.8	78.0	30.1	1.1	H. C.	73,000,000
	20	0.0008	8.0	72.7	36.9	1.1	H. C.	7,500,000
Aerated with air con- taining 20 per cent CO ₂	5	0.0015	7.6	74.5	27.1	0.9	M. C.	110,000,000
	9	0.001	7.4	79.2	30.5	0.95	H. C.	91,000,000
	20	0.001	7.6	71.9	36.2	1.0	H. C.	21,000,000
Medium			7.5	64.0	10.0			

M. C. = moderate crust; H. C. = heavy crust.

bated under atmospheres containing 5, 10 or 20 per cent carbon dioxide.

Hydrogen ion concentration (chart 6). The final pH values of the control cultures and the culture incubated under gas mixtures containing 5, 10 and 20 per cent CO₂ were 9.0, 8.2, 8.0 and 7.6, respectively. If the favorable effects of an increased carbon dioxide tension on toxin production and growth is due principally

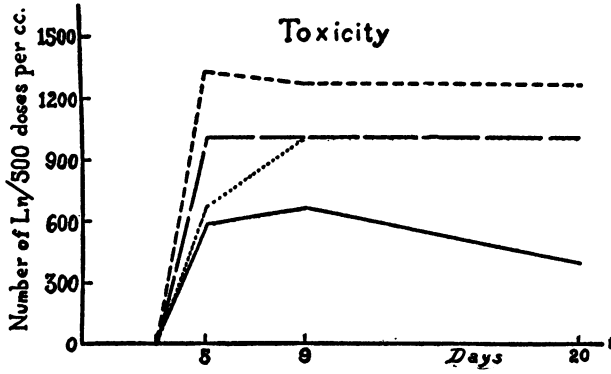


CHART 5

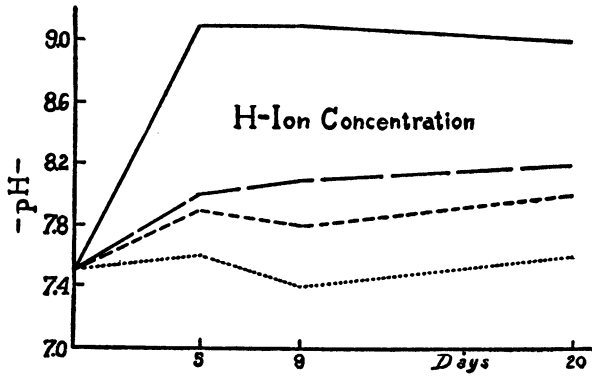


CHART 6

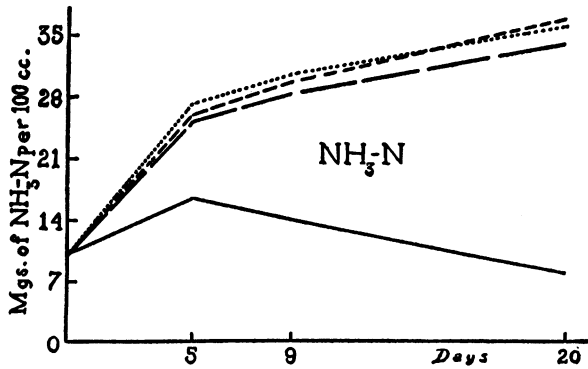


CHART 7

— control.
 - - - aerated with 5 per cent CO₂.

to a regulation of the reaction of the culture medium, these results would show that a maintained hydrogen ion concentration of from pH 7.8 to 8.0 is the optimum for growth and toxin accumulation.

Ammonia nitrogen (chart 7). The accumulation of ammonia in the control cultures (unaerated flasks) was more gradual and never as great as in the other cultures; furthermore, a slow but continuous decrease in ammonia content occurred between the end of the period of maximum growth and the end of the 20 day incubation period. Apparently, under the usual conditions of

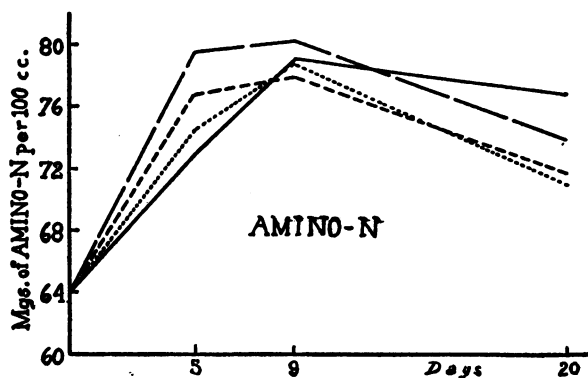


CHART 8

----- aerated with 10 per cent CO₂.
 aerated with 20 per cent CO₂.

artificial cultivation, ammonia passed off into the outside atmosphere, but was retained in the cultures aerated with an atmosphere containing from 3 to 20 per cent carbon dioxide. The presence of appreciable numbers of viable cells during the entire twenty-day incubation period in cultures grown under an increased carbon dioxide tension probably explains the continuous increase in ammonia in such cultures.

Amino nitrogen (chart 8). Increase in amino nitrogen was more rapid in cultures aerated with atmospheres containing from 5 to 20 per cent carbon dioxide than in the control cultures. Subsequent decrease in amino nitrogen content was more marked in the former than in the latter, a condition which shows that an

increased carbon dioxide tension within certain limits increases either the amount or the activity of the peptolytic and deaminizing enzymes of *C. diphtheriae*.

Plate count (chart 9). The number of viable organisms remaining after the period of maximum growth was considerably greater in the cultures aerated with atmospheres containing either 5, 10 or 20 per cent carbon dioxide, particularly the last, than in the control cultures. The plate count for the control cultures approached zero on the fifth day of growth; as no counts were

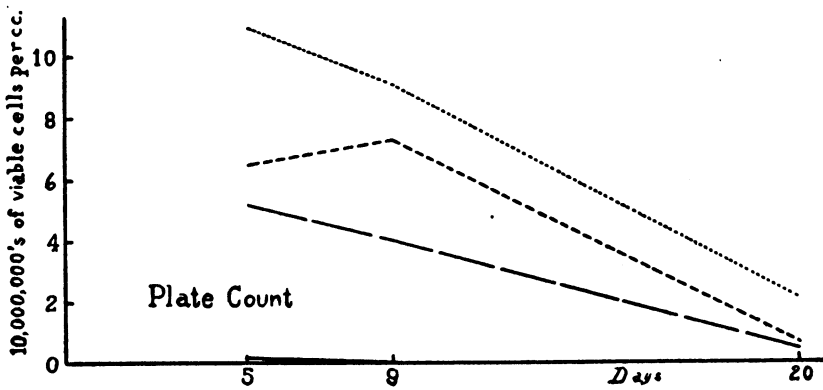


CHART 9

—— control.
 — aerated with 5 per cent CO₂.
 - - - aerated with 10 per cent CO₂.
 aerated with 20 per cent CO₂.

made before the fifth day, the curve for the control cultures is barely visible in chart 9.

Bacterial sediment. More bacterial sediment was produced in the cultures aerated with an atmosphere containing from 5 to 10 per cent carbon dioxide than in any of the other cultures.

Remarks. From these results the conclusion may be drawn that from 5 to 10 per cent carbon dioxide within the culture flasks is the optimum concentration for growth and toxin accumulation.

Growth and toxin production in bouillon prepared with Difco-Bacto Peptone

The character of the "peptone" employed in the production of diphtheria toxin has been known for some time to have a direct bearing on the quantity of toxin obtained in culture filtrates. Consequently, many attempts have been made to determine why certain "peptones" favor abundant toxin accumulation while other "peptones" yield little or no toxin, even though they support abundant growth of *C. diphtheriae*. The results of past investigation have led to the development of the theory that simple nitrogenous compounds such as amino-acids are necessary and sufficient for growth, while more complex bodies, probably proteoses, are essential for toxin accumulation.

As to how these proteose fractions come into play in toxin formation has been a matter of considerable speculation. Dernby and Walbum (1923) suggest that the diphtheria organism elaborates an enzyme which converts certain proteoses present in the culture medium into the specific toxic substance found in cultures of *C. diphtheriae*. Kligler (1917) and Bunker (1919) intimate that "peptones" of high proteose content are necessary for toxin production because of their buffering effect on the reaction of the culture medium.

The experiments already described show that it is possible to regulate the hydrogen ion concentration of the culture medium (within limits) by increasing the carbon dioxide tension over the culture. Difco-Proteose Peptone is especially adapted for toxin production, and is known to possess a high proteose content. On the other hand, Difco-Bacto Peptone, while it will support abundant growth of most bacteria, is known to be quantitatively lacking in higher protein derivatives.⁵ Therefore, in order to determine whether or not the proteose fractions of a "peptone" are necessary for toxin formation purely because of their buffering action, the following experiment was carried out, employing Difco-Bacto Peptone in place of Difco-Proteose Peptone.

⁵ A chemical analysis of these two peptones has been reported by McAlpine and Brigham (1928).

Twelve flasks of bouillon were prepared with Difco-Bacto Peptone. These were inoculated, each with 1 cc. of an acclimated culture of *C. diphtheriae*, and divided into 4 groups, 3 flasks in each group. Group I served as the control, while Groups

TABLE 6
Giving the results obtained with Difco Bacto Peptone under different concentrations of carbon dioxide

CULTURE	NUM- BER OF DAYS INCUBATED	Ln/500 DOSE	pH	AMINO- N IN 100 CC.	NH ₂ -N IN 100 CC.	NUM- BER OF CUBIC CENTI- METERS SEDIM- ENT IN 100 CC.	CHARAC- TER OF GROWTH	VIABLE CELLS PER CUBIC CENTI- METER
		cc.		mgm.	mgm.			
Set I. Incubated under ordinary conditions	5	>0.05	9.0	77.2	20.4	0.8	L. C.	30,000,000
	9	0.05	9.0	78.1	15.4	0.8	L. C.	12,000
	20	>0.05	8.8	80.1	14.1	0.7	Br. C.	10,000
Set II. Aerated with air containing 5 per cent CO ₂	5	>0.05	7.8	71.5	27.1	0.8	M. C.	40,000,000
	9	0.05	8.0	75.2	30.0	1.0	M. C.	30,000,000
	20	0.05	8.0	71.0	31.2	1.0	M. C.	3,100,000
Set III. Aerated with air containing 10 per cent CO ₂	5	0.03	7.6	72.6	30.0	0.9	M. C.	65,000,000
	9	0.03	7.6	74.4	30.8	1.0	M. C.	24,000,000
	20	0.03	7.7	70.5	31.4	1.0	M. C.	5,500,000
Set IV. Aerated with air containing 20 per cent CO ₂	5	>0.05	7.3	70.8	28.6	0.8	L. C.	75,000,000
	9	>0.05	7.2	67.7	31.1	0.95	M. C.	40,000,000
	20	>0.05	7.2	76.1	32.1	1.0	M. C.	10,000,000
Medium			7.5	68.8	8.0			

Note: (>) means that the Ln/500 dose is greater than the amount of toxin given in the table. The amounts of toxin preceded by this sign failed completely to give a reaction.

L. C. = light crust; M. C. = moderate crust; Br. C. = broken crust partly sunk.

II, III and IV were aerated with atmospheres containing 5, 10 and 20 per cent carbon dioxide, respectively. One flask was removed from each group after 5, 9 and 20 days of incubation.

The results are recorded in table 6.

Toxicity. While all cultures grown on Difco-Bacto Peptone

were but feebly toxic, toxin formation was most rapid and abundant in cultures aerated with an atmosphere containing 10 per cent carbon dioxide. No decrease in toxicity occurred in these cultures on prolonged incubation. In the control cultures, a trace of toxin appeared on the ninth day and disappeared after 20 days incubation. No toxin was detectable at any time in the cultures aerated with an atmosphere containing 20 per cent carbon dioxide, probably because the hydrogen ion concentration of these cultures was unfavorable for toxin accumulation (pH 7.2).

Growth. Under similar conditions of incubation, less growth was obtained with Difco-Bacto Peptone than with Difco-Proteose Peptone, as shown by bacterial sediment determinations, even though surface growth was abundant in nutrient broth prepared with either "peptone." There was not enough difference in growth, however, in the two media to explain the marked difference in toxin accumulation. Maximum growth occurred in cultures grown under from 5 to 10 per cent carbon dioxide.

Ammonia nitrogen. Ammonia production was more rapid in broth made with Difco-Bacto Peptone than in broth made with Difco-Proteose Peptone. However, the total amount produced was greater in broth prepared with the latter. Maximum ammonia accumulation occurred in cultures aerated with an atmosphere containing either 5 or 10 per cent carbon dioxide.

Hydrogen ion concentration. The hydrogen ion concentration of cultures subjected to 5, 10 and 20 per cent carbon dioxide was maintained at pH 8.0, pH 7.6 and pH 7.2, respectively.

Amino nitrogen. An increase in amino nitrogen occurred in all cultures during the first nine days of growth, which was followed by a gradual decrease throughout the remainder of the incubation period in cultures aerated with an atmosphere containing from 5 to 10 per cent carbon dioxide. In some of the control cultures the usual decrease in amino nitrogen content failed to occur, as shown in table 6. This condition was the exception rather than the rule.

A comparison of the change in amino nitrogen content of cultures grown in broth prepared with Difco-Bacto Peptone and in broth prepared with Difco-Proteose Peptone is given in chart

10. A relatively slight increase in amino nitrogen occurred in cultures in which Difco-Bacto Peptone was employed, as compared with a marked increase in cultures grown in Difco-Proteose Peptone broth. Apparently protein derivatives which can be broken down into amino acids by the peptolytic enzymes of *C. diphtheriae* are present in much smaller amounts in Difco-Bacto Peptone than in Difco-Proteose Peptone.

Remarks. From these results it appears that the proteose fractions of certain "peptones" suitable for diphtheria toxin production are primarily necessary for reasons other than their buffering effect on the reaction of the culture medium. While a

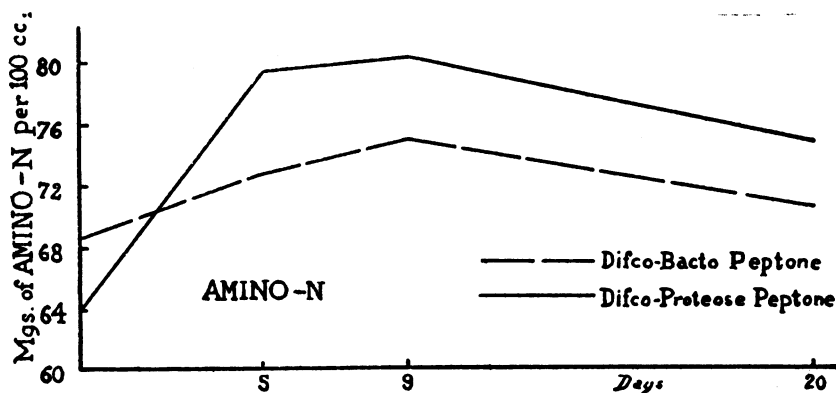


CHART 10

rich supply of proteose is not essential for development, growth is more luxuriant in a medium containing relatively large amounts of higher protein derivatives.

The influence of different oxygen tensions on growth and toxin production

An abundant oxygen supply has been regarded as essential for toxin production for some time, although few attempts have been made to determine the effect of different oxygen tensions on the growth and toxin production of *C. diphtheriae*.

Wherry (1917) found that the oxygen supply is an important factor in the evolution of the larger types of *C. diphtheriae*, low

oxygen tensions favoring the formation of barred forms, and distinctly aerobic conditions favoring the formation of small solid-staining types. Diphtheria organisms re-isolated from guinea pigs which had been injected with virulent strains of *C. diphtheriae* grew better at lowered oxygen tension than under ordinary atmospheric conditions.

Lorentz (1923) observed that colonies of *C. diphtheriae* grown on Loeffler's serum under pure oxygen were smaller and fewer in number than those grown under ordinary aerobic conditions. Under an atmosphere of pure oxygen the individual cells were

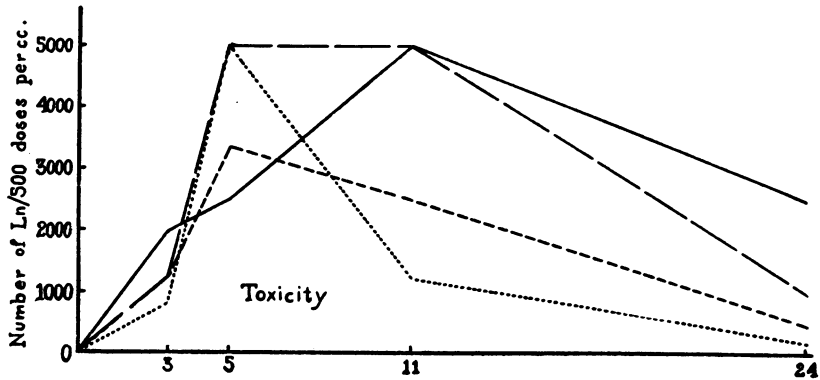


CHART 11. IN THE ABSENCE OF ADDED CO₂

shorter and thicker than in the control cultures, and showed a marked reduction in polar granules. He further observed that cultures grown under pure oxygen gas were more toxic than those grown under pure carbon dioxide gas.⁶

As it is possible to maintain fairly constant oxygen and carbon dioxide tensions in culture flasks by means of the aeration system described in this paper, a study was made of the influence of different oxygen tensions in the presence and in the absence of added carbon dioxide upon growth and toxin production of *C. diphtheriae*.

⁶ Diphtheria toxin is never found in media having an acid reaction. As our results have shown, more than 20 per cent carbon dioxide in the atmosphere over the culture inhibits toxin formation through the resulting increase in hydrogen ion concentration of the medium.

A. Cultures grown under increased oxygen tensions. 1. In the absence of added CO₂. Sixteen flasks of broth were prepared in

TABLE 7
Showing influence of increased oxygen in the absence of added carbon dioxide

CULTURE	NUM- BER OF DAYS INCUBATION	Ln/500 DOSE	pH	NH ₃ -N IN 100 CC. OF CUL- TURE	SEDI- MENT IN 100 CC. OF CUL- TURE	CHARAC- TER OF GROWTH	NUMBER OF VIA- BLE CELLS PER CUBIC CENTIMETER OF CULTURE
		cc.		mgm.	cc.		
Group I. Controls. Incubated under ordinary conditions	3	0.0005	7.9	20.3	0.8	M. C.	135,000,000
	5	0.0004	9.1	23.0	1.2	M. C.	1,500,000
	11	0.0002	9.1	12.6	1.1	M. C.	No colony 1/10 plate
	24	0.0004	9.0	8.8	1.1	M. C.	No colony 1/10 plate
Group II. Aerated with gas-mixture containing 21 per cent oxygen	3	0.0008	8.0	24.5	0.7	M. C.	117,000,000
	5	0.00018	8.4	24.0	0.9	M. C.	6,000,000
	11	0.0002	8.4	26.8	1.0	M. C.	No colony 1/10 plate
	24	0.001	9.1	15.1	1.0	Br. C.	No colony 1/10 plate
Group III. Aerated with a gas-mixture containing 30 per cent oxygen	3	0.0008	8.1	21.0	0.5	L. C.	83,000,000
	5	0.0003	9.0	25.7	0.8	M. C.	300,000
	11	0.0004	9.1	22.7	1.0	M. C.	No colony 1/10 plate
	24	0.002	9.1	13.9	1.0	M. C.	No colony 1/10 plate
Group IV. Aerated with a gas-mixture containing 50 per cent oxygen	3	0.0012	8.0	20.5	0.5	L. C.	60,000,000
	5	0.0002	8.8	27.0	1.0	M. C.	2,000,000
	11	0.0008	9.1	19.2	1.0	M. C.	No colony 1/10 plate
	24	0.005	9.1	12.4	1.0	Br. C.	No colony 1/10 plate
Medium			7.5	9.5			

M. C. = Moderate crust; L. C. = light crust; Br. C. = broken crust partly sunk.

the usual way with Difco-Proteose Peptone, and inoculated in the adopted fashion. Four of the flasks were incubated under

ordinary atmospheric conditions as controls; 4 were aerated with CO₂-free atmosphere, containing 21 per cent oxygen; 4 were similarly exposed to 30 per cent oxygen; and 4 to a CO₂-free atmosphere containing 50 per cent oxygen. One flask was removed from each group after three, five, eleven and twenty-four days incubation. The usual determinations were made on each culture.

The results are recorded in table 7.

Toxicity (chart 11). Toxin formation was most rapid during the first three days of incubation in the cultures grown under ordinary atmospheric conditions. This was undoubtedly due to the fact that CO₂-free atmospheres were passed over the aerated cultures. However, after initial growth was once started, this condition was reversed, the five-day aerated cultures being more toxic than the five-day controls. Maximum potency was attained in the aerated cultures on the 5th day, and in the controls on the 11th day.

A rapid decrease in toxin content of the aerated cultures occurred immediately after maximum potency was reached. The rate of decrease was greatest in cultures treated with 50 per cent oxygen, slightly less rapid in those aerated with an atmosphere containing 30 per cent oxygen, still less rapid in cultures under 21 per cent oxygen, and least rapid in the control cultures. The number of viable organisms per cubic centimeter of test culture decreased rapidly after the period of maximum growth, both in the control and in the aerated cultures.

2. *In the presence of 5 per cent CO₂*. The preceding experiment was repeated, except that test atmospheres of 5 per cent carbon dioxide were employed with the 21, 30 and 50 per cent oxygen, respectively.

The results are recorded in table 8.

Toxicity (chart 12). Toxin accumulation was more rapid and abundant in the cultures aerated with an atmosphere containing 5 per cent carbon dioxide and 21 per cent oxygen than in either the controls or the cultures exposed to 5 per cent carbon dioxide and either 30 or 50 per cent oxygen.

No decrease in toxicity occurred in cultures aerated with a

gas-mixture containing 5 per cent carbon dioxide and 21 per cent oxygen on prolonged cultivation. A slight loss in potency occurred at the end of twenty-four days incubation in cultures

TABLE 8
Showing the influence of increased oxygen supply in the presence of 5 per cent carbon dioxide

CULTURE	NUM- BER OF DAYS INCUBATION	Ln/500 DOSE	pH	NH ₄ -N IN 100 CC. OF CUL- TURE	SEDI- MENT IN 100 CC. CUL- TURE	CHARAC- TER OF GROWTH	NUMBER OF VIABLE CELLS PER CUBIC CENTIMETER OF CULTURE
		cc.		mgm.	cc.		
Group I. Controls. Incubated under ordinary atmospheric conditions	3	0.0005	7.9	20.3	0.6	M. C.	120,000,000
	5	0.0004	9.1	21.7	0.9	M. C.	1,000,000
	10	0.0003	9.1	14.0	1.0	M. C.	No colony 1/10 plate
	24	0.0015	9.0	6.0	0.9	M. C.	No colony 1/10 plate
Group II. Aerated with a gas-mixture containing 20 per cent oxygen and 5 per cent CO ₂	3	0.0002	8.0	23.0	1.0	H. C.	150,000,000
	5	0.0002	8.0	30.1	1.0	H. C.	33,000,000
	10	0.0002	8.1	33.4	1.0	H. C.	64,000,000
	24	0.0002	8.1	36.0	1.0	H. C.	1,600,000
Group III. Aerated with a gas-mixture containing 30 per cent oxygen and 5 per cent CO ₂	3	0.0005	8.0	21.7	0.95	H. C.	118,000,000
	5	0.0002	8.0	31.7	1.0	H. C.	18,000,000
	10	0.0002	8.0	32.6	1.0	H. C.	2,000,000
	24	0.00025	8.1	33.0	1.1	H. C.	500,000
Group IV. Aerated with a gas-mixture containing 50 per cent oxygen and 5 per cent CO ₂	3	0.0008	7.6	20.3	0.6	M. C.	135,000,000
	5	0.0002	7.8	30.5		M. C.	62,000,000
	10	0.0002	8.1	31.5	0.8	M. C.	1,200,000
	24	0.0004	8.1	32.7	0.9	M. C.	40,000
Medium			7.5	10.1			

M. C. = moderate crust; H. C. = heavy crust.

grown under an increased oxygen tension; however, this decrease in toxicity was not nearly as great in the presence, as in the absence, of added carbon dioxide.

Growth. The number of viable organisms remaining after the

period of maximum growth was highest in cultures grown under an atmosphere containing 5 per cent carbon dioxide and 21 per cent oxygen, although the number remaining in all cultures aerated with test atmospheres containing added carbon dioxide was appreciably greater than the number remaining in the control cultures.

Sediment determinations and the character of the surface growth showed that growth was most rapid and abundant in cultures aerated with an atmosphere containing 5 per cent carbon

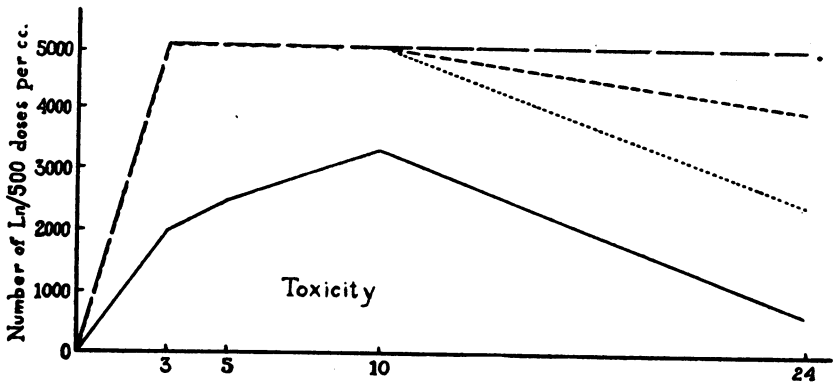


CHART 12. IN THE PRESENCE OF 5 PER CENT CO₂

— control.
 — oxygen tension—21 per cent.
 - - - oxygen tension—30 per cent.
 oxygen tension—50 per cent.

dioxide and 21 per cent oxygen, while growth was retarded and least abundant in cultures grown under a gas-mixture containing 5 per cent carbon dioxide and 50 per cent oxygen.

Remarks. An increased oxygen tension above 21 per cent, in the absence of added carbon dioxide, retards the growth of *C. diphtheriae* and causes increased irregularity in toxin accumulation. These unfavorable conditions are offset to a large degree by the presence of added carbon dioxide.

From the results presented, the conclusion may be drawn that 21 per cent oxygen in the atmosphere over *C. diphtheriae* cultures is more favorable for growth and toxin accumulation than higher oxygen tensions.

B. Cultures grown under reduced oxygen tensions. Obviously, the oxygen concentration prevailing under natural conditions of growth for *C. diphtheriae* is less, and the carbon dioxide tension greater, than that prevailing under ordinary aerobic conditions of artificial cultivation. At the same time, the results of the foregoing experiment show that an increased oxygen tension above 21 per cent does not favor growth and toxin accumulation. In view of these facts, the following experiment was conducted for the purpose of determining the optimum and minimum oxygen tensions for growth and toxin production, and to determine whether or not added carbon dioxide influences growth and toxin accumulation in cultures grown under reduced oxygen supply.

Lowered oxygen tensions were obtained by collecting the required amounts of air, carbon dioxide and nitrogen gas⁷ over water in the gas supply bottles of the aeration systems. Samples of the resulting gas-mixtures were analyzed by means of an Orsat-Lunge Gas Apparatus.

1. *In the absence of added CO₂.* Sixteen flasks of meat-infusion-peptone broth were inoculated with *C. diphtheriae*, divided equally into 4 groups, and placed in the incubator. Three of the groups were aerated with atmospheres containing 15, 10 and 5 per cent oxygen, respectively, while the remaining group was grown under ordinary atmospheric conditions and served as the control. One flask was removed from each group after three, five, ten and twenty days incubation.

The results are tabulated in table 9.

Toxicity (chart 13). During the first three days of growth the rate of toxin formation in the cultures aerated with atmospheres containing either 10 or 15 per cent oxygen paralleled that of the control cultures, while toxin production was somewhat retarded in cultures aerated with a gas mixture containing 5 per cent oxygen.

Maximum potency was reached in all cultures on the fifth day of incubation. Toxin accumulation was greatest in cultures

⁷ The nitrogen used for this purpose was prepared in the laboratory as needed, by heating the proper mixture of ammonium sulphate and sodium nitrite in aqueous solution.

aerated with an atmosphere containing 15 per cent oxygen, slightly less in cultures supplied with 10 per cent oxygen, still

TABLE 9
Showing the influence of reduced oxygen tension in the absence of added carbon dioxide

CULTURE	DAYS INCUBATION	Ln/500 DOSE	pH	NH ₃ -H IN 100 CC.	SEDIMENT IN 100 CC. CULTURE	CHARACTER OF GROWTH	VIABLE CELLS PER CUBIC CENTIMETER CULTURE
		cc.		mgm.	cc.		
Group I. Controls, grown under ordinary atmospheric conditions	3	0.0003	8.6	20.1	0.9	L. C.	250,000,000
	5	0.00025	8.8	21.7	0.9	M. C.	300,000
	10	0.00025	9.0	13.0	0.8	M. C.	No colony 1/10 plate
	20	0.0005	9.0	8.7	0.8	M. C.	No colony 1/10 plate
Group II. Aerated with CO ₂ -free atmosphere containing 15 per cent oxygen	3	0.0003	7.8	19.2	0.9	L. C.	250,000,000
	5	0.00015	8.6	22.2	1.0	H. C.	47,000,000
	10	0.0002	8.9	23.1	1.0	H. C.	No colony 1/10 plate
	20	0.0003	9.1	14.7	0.9	H. C.	No colony 1/10 plate
Group III. Aerated with CO ₂ -free atmosphere containing 10 per cent oxygen	3	0.00025	7.6	20.3	1.0	L. C.	300,000,000
	5	0.00018	8.2	22.0	1.0	H. C.	53,000,000
	10	0.00025	9.0	20.3	0.9	M. C.	No colony 1/10 plate
	20	0.0003	9.1	16.4	0.85	M. C.	No colony 1/10 plate
Group IV. Aerated with CO ₂ -free atmosphere containing 5 per cent oxygen	3	0.001	7.1	18.6	0.6	L. F.	210,000,000
	5	0.0002	8.2	21.4	1.0	H. C.	113,000,000
	10	0.0002	8.8	21.4	0.8	M. C.	No colony 1/10 plate
	20	0.0002	8.9	11.6	0.9	M. C.	No colony 1/10 plate
Medium			7.5	10.6			

L. C. = light crust; M. C. = moderate crust; H. C. = heavy crust; L. F. = light film.

less in cultures grown under a gas mixture containing 5 per cent oxygen, and least in the control cultures.

Decrease in toxicity after the period of maximum potency was inversely proportional to the oxygen tensions in the culture flasks. No noticeable decrease in toxicity occurred in the twenty-day cultures grown under an atmosphere containing 5 per cent oxygen; there was a marked decrease in toxicity in cultures grown under an atmosphere containing 10 per cent oxygen; the decrease was still more pronounced in cultures aerated with a gas mixture containing 15 per cent oxygen, and greatest in the control cultures.

Growth. Growth was most rapid and abundant in cultures aerated with atmospheres containing either 10 or 15 per cent

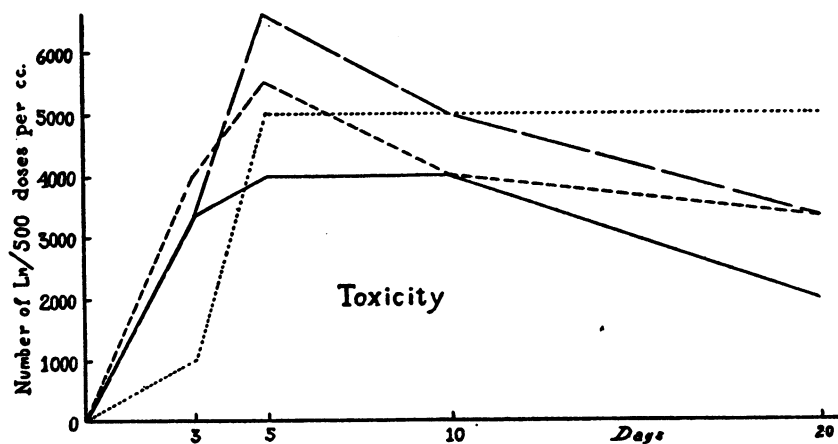


CHART 13. IN THE ABSENCE OF ADDED CO₂

oxygen, and least rapid in cultures grown under a gas mixture containing 5 per cent oxygen.

The pellicles formed on cultures supplied with 5 or 10 per cent oxygen were distinctly membranous in appearance, and were disintegrated with considerably more difficulty than the pellicles formed under ordinary or increased oxygen tensions.

2. *In the presence of 5 per cent CO₂.* The preceding experiment was repeated except that the gas supply bottles of the three aeration systems contained 5 per cent carbon dioxide; 5, 10 and 15 per cent oxygen were again used. The 4 control cultures were incubated under ordinary atmospheric conditions.

The results are expressed in table 10.

Toxicity (charts 13 and 14). Toxin accumulation was decidedly more rapid and abundant in cultures aerated with test

TABLE 10
Showing the influence of reduced oxygen tension in the presence of 5 per cent carbon dioxide

CULTURE	DAYS INCUBATION	Ln/500 DOSE	pH	NH ₃ -N IN 100 CC.	SEDIMENT IN 100 CC. CULTURE	CHARACTER OF GROWTH	VIABLE CELLS PER CUBIC CENTIMETER CULTURE
		cc.		mgm.	cc.		
Group I. Controls. Grown under ordinary conditions	3	0.0005	8.6	20.8	0.8	M. C.	
	5	0.0003	8.7	22.7	0.8	M. C.	180,000
	10	0.00025	8.6	17.7	0.7	M. C.	No colony 1/10 plate
	20	0.002	8.7	12.0	0.7	M. C.	No colony 1/10 plate
Group II. Aerated with atmosphere containing 15 per cent oxygen and 5 per cent CO ₂	3	0.0002	7.8	26.7	1.2	H. C.	370,000,000
	5	0.00015	8.1	31.8	1.2	H. C.	60,000,000
	10	0.00015	8.1	29.0	1.0	H. C.	23,000,000
	20	0.00015	8.1	32.0	1.1	H. C.	3,000,000
Group III. Aerated with atmosphere containing 10 per cent oxygen and 5 per cent CO ₂	3	0.0002	7.8	27.0	1.0	H. C.	310,000,000
	5	0.00015	8.0	30.4	1.1	H. C.	70,000,000
	10	0.00015	8.1	30.7	1.1	H. C.	10,000,000
	20	0.00015	8.1	31.0	1.1	H. C.	5,000,000
Group IV. Aerated with atmosphere containing 5 per cent oxygen and 5 per cent CO ₂	3	0.00022	7.8	27.0	0.9	H. C.	210,000,000
	5	0.00015	8.0	30.1	1.0	H. C.	140,000,000
	10	0.00015	8.1	30.7	1.1	H. C.	12,000,000
	20	0.00015	8.1	30.1	1.0	H. C.	No count
Medium			7.5	11.0			

M. C. = moderate crust; H. C. = heavy crust.

atmospheres containing 5 per cent carbon dioxide and either 5, 10 or 15 per cent oxygen than in the control cultures or cultures aerated with CO₂-free atmospheres containing 5, 10 or 15 per cent oxygen.

Maximum potency was attained in all 3 groups of aerated cultures on the fifth day of growth and was maintained throughout the entire twenty-day incubation period.

The most potent culture filtrates obtained during the course of this investigation were obtained from cultures grown under 5 per cent carbon dioxide and either 10 or 15 per cent oxygen.

Growth. Heavy crusts were formed on the surface of all 3 groups of aerated cultures during the first 3 days of growth; however, the greatest amount of bacterial sediment was present

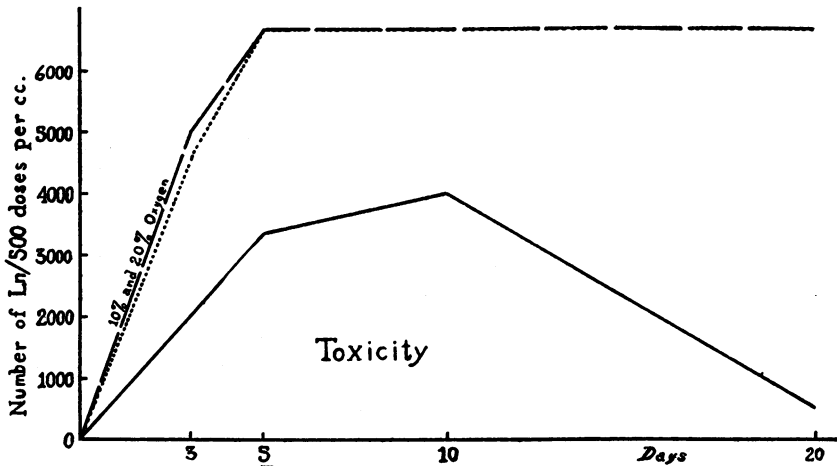


CHART 14. IN THE PRESENCE OF 5 PER CENT CO_2

- control.
- oxygen tension—15 per cent.
- oxygen tension—10 per cent.
- oxygen tension—5 per cent.

in cultures (shaken) aerated with a gas mixture containing 5 per cent carbon dioxide and 15 per cent oxygen. The number of viable organisms remaining after the period of maximum growth continued at a relatively high figure throughout the twenty-day incubation period.

3. *Cultures aerated with an atmosphere containing 1.5 per cent oxygen, with and without added CO_2 .* In view of the fact that growth and toxin production were but slightly influenced in cultures grown under as little as 5 per cent oxygen, cultures were

aerated with atmospheres containing 1.5 per cent oxygen, with and without added carbon dioxide. Six flasks of broth were inoculated and divided into 3 groups, 2 flasks in each group. Group I was aerated with a CO₂-free atmosphere; Group II was aerated with an atmosphere containing 1.5 per cent oxygen and 5 per cent carbon dioxide and Group III was incubated under ordinary atmospheric conditions, as a control. The results are recorded in table 11.

TABLE 11
Showing the effect of aeration with 1.5 per cent oxygen with and without added carbon dioxide

CULTURE	DAYS INCUBATION	Ln/500 DOSE	pH	BACTERIAL SEDIMENT IN 100 CC. CULTURE	CHARACTER OF GROWTH	VIABLE CELLS PER CUBIC CENTIMETER OF CULTURE
Group I. Grown under ordinary atmospheric conditions	5	0.0003	8.7	cc. 0.9	M. C.	250,000,000
	15	0.0004	9.0	0.9	M. C.	0
Group II. Aerated with atmosphere containing 1.5 per cent O ₂ and no CO ₂	5	>0.05	7.0	0.15	S. G.	350,000,000
	15	0.0005	7.8	0.5	L. C.	300,000,000
Group III. Aerated with atmosphere containing 1.5 per cent O ₂ and 5 per cent CO ₂	5	>0.05	6.8	0.10	S. G.	150,000,000
	15	0.002	6.8	0.3	S. G.	230,000,000

M. C. = moderate crust; S. G. = scant growth; L. C. = light membranous crust.

Toxin production and growth were greatly retarded in cultures supplied with as little as 1.5 per cent oxygen; this retardation was greatest in the presence of 5 per cent carbon dioxide. The aerated cultures harvested on the fifth day were practically non-toxic; however, after fifteen days of incubation measurable amounts of toxin were present in both sets of aerated cultures. The fifteen-day cultures aerated with a gas mixture containing 1.5 per cent oxygen and no added carbon dioxide were several times more

potent than the culture subjected to the same amount of oxygen, and 5 per cent carbon dioxide.

Apparently the presence of 5 per cent carbon dioxide in the gas mixture passed over the cultures prevented the usual oxidation of the organic acids which, as shown by Wolf (1922), are formed from amino acids during the initial growth of *C. diphtheriae* in sugar-free peptone broth. As a result the hydrogen ion concentration of the medium remained below pH 7.0 during the 15 day incubation period, thereby inhibiting growth and toxin formation to a greater extent than in the cultures aerated with 1.5 per cent oxygen, without added carbon dioxide.

Remarks. The conclusion may be drawn from the data presented here that the ideal gaseous environment for growth and toxin accumulation is supplied by an atmosphere containing 5 per cent carbon dioxide and 15 per cent oxygen.

4. *Cultures grown in the presence of a limited oxygen supply.* *C. diphtheriae*, when grown under aerobic conditions on a carbohydrate-free medium, utilizes atmospheric oxygen and gives off carbon dioxide. In order to determine the quantity of oxygen necessary for normal growth and toxin formation, 90 cc. cultures were grown in sealed 3-liter jars with and without added carbon dioxide. In both cases the toxicity of the resulting culture filtrates was greater than the filtrates obtained from the control cultures which were grown under ordinary atmospheric conditions (without seal). Toxin formation was slightly more rapid in the jars containing 3 per cent carbon dioxide at the start than in the jars having ordinary air. The original oxygen content within the sealed jars was reduced from 20 per cent to about 10 per cent, representing a consumption of approximately 300 cc. of oxygen (at 20°C. and 760 mm. pressure) by 90 cc. of broth culture. The carbon dioxide content of the jars containing 3 per cent carbon dioxide at the outset was increased to approximately 10 per cent, while that of the jars originally containing ordinary air was found to be 7 per cent.

That atmospheric oxygen is necessary for toxin formation was shown by growing *C. diphtheriae* in sealed 500 cc. Erlenmeyer flasks containing 90 cc. of medium and ordinary air. Less toxin

was formed under this condition than was formed in the control cultures, and when much larger containers were used. The available supply of oxygen contained in the sealed flasks was practically exhausted.

GENERAL DISCUSSION

The experiments reported herein were undertaken for the purpose of determining the influence of gaseous environment on the growth and toxin production of *C. diphtheriae*, with special reference to carbon dioxide.

From the start, the results indicated that the carbon dioxide tension within the culture flask has a direct bearing on the rate of growth and the amount of toxin produced by *C. diphtheriae*. Aeration of cultures with an atmosphere containing 5 per cent carbon dioxide and from 10 to 20 per cent oxygen resulted in an increased growth momentum, and more rapid and abundant toxin formation, than occurred under ordinary conditions of artificial cultivation. Furthermore, with a given medium, culture filtrates were strikingly uniform in potency. No decrease in toxicity occurred in these aerated cultures on prolonged cultivation at 37°C. While an increased carbon dioxide tension (within the limits mentioned) favored growth and toxin accumulation, greater concentrations than 10 per cent in the atmosphere within the culture flasks increased the hydrogen ion concentration of the medium beyond the optimum for growth and toxin production.

In regard to the oxygen tension necessary for toxin formation, the results have shown that, so long as the concentration of oxygen in the atmosphere within the culture flasks did not fall below 5 per cent, no inhibition of toxin production occurred. As a matter of fact, a slightly reduced oxygen tension appeared to enhance, rather than to retard both growth and toxin accumulation.

The favorable influence of carbon dioxide on growth and toxin formation may depend upon one or more of the following conditions: (1) As shown by Valley and Rettger (1927), carbon dioxide is necessary for the initial growth of *C. diphtheriae*, hence the presence of from 3 to 10 per cent carbon dioxide in the atmosphere

within the culture flasks induces early initial cell development. (2) Soon after the maximum growth period sets in, the reaction of the medium in non-carbohydrate cultures grown under aerobic conditions in the absence of added carbon dioxide becomes sufficiently alkaline to be bacteriostatic, if not bactericidal. On the other hand, if the atmosphere in the culture flask contains from 3 to 10 per cent carbon dioxide, the reaction never becomes unfavorable for cell multiplication and toxin accumulation. The importance of preventing the reaction from becoming too alkaline has been demonstrated by Bunker (1919) who was unable to obtain potent toxins in cultures in which the pH value exceeded 8.25.

Similar results have been reported by Dernby and David (1921) who found that the limiting hydrogen ion concentrations for growth were pH 6.0 and pH 8.3. (3) A recent paper by Locke and Main (1928) has indicated that toxin is not accumulated unless there is a definite growth momentum. That growth momentum is increased by an increased carbon dioxide tension has been demonstrated in the present paper. This condition may explain, in part, increased toxin accumulation in cultures aerated with atmospheres containing from 3 to 10 per cent carbon dioxide. (4) An increased carbon dioxide tension prevents the destruction of diphtheria toxin once it is formed. An explanation of the mechanism responsible for the preserving effect of carbon dioxide on diphtheria toxin will be presented in another paper.

The data presented show that the carbon dioxide content of *C. diphtheriae* cultures has considerable influence on growth and on the amount of toxin present at the time of harvesting. This condition, together with the fact that the diphtheria organism produces appreciable quantities of carbon dioxide when grown in sugar-free broth (Wolf, 1922; Apt and Loiseau, 1925), should explain to a considerable degree the lack of uniformity in the toxicity of cultures grown under ordinary atmospheric conditions. That is to say, the rate of destruction of diphtheria toxin varies in different culture flasks containing the same lot of medium, due to the difference in rate at which carbon dioxide

leaves the culture medium after the period of maximum growth. The rate of outward diffusion of the gas from the culture grown under ordinary atmospheric conditions depends upon the character of the medium, size and shape of the culture flask, density of the cotton plugs, and the conditions existing inside the incubator, such as the number of cultures present and the facilities for ventilation.

Dernby and Walbum (1923), have suggested that destruction of toxin in *C. diphtheriae* cultures may be due to the "peptolytic" action of enzymes elaborated by the diphtheria organism. Such an explanation appears to be discredited by the lack of toxin destruction in cultures grown under atmospheres containing from 3 to 10 per cent carbon dioxide.

As will be shown in a following paper, destruction of toxin which usually follows the period of maximum toxicity in cultures grown under aerobic conditions in the absence of added carbon dioxide, is probably due in a large degree to oxidation of the toxin molecule; in the presence of suitable amounts of carbon dioxide this oxidation process is largely or entirely prevented.

SUMMARY

1. The intracutaneous test, as employed in this investigation, was found to be a reliable and economical means of determining the approximate toxicity of *C. diphtheriae* culture-filtrates.

2. Aeration of broth cultures of *C. diphtheriae* with atmospheres containing from 3 to 10 per cent carbon dioxide and from 5 to 50 per cent oxygen resulted in increased growth and toxin production.

3. Aeration of broth cultures with ordinary air or CO₂-free atmospheres containing from 10 to 50 per cent oxygen resulted in marked irregularity in growth and in the toxin content of *C. diphtheriae* cultures. The data obtained show that the higher the oxygen content of the atmospheres passed over the cultures the greater is the irregularity in toxin content of the cultures at the time of harvesting. The rate of destruction of the toxin once it was formed was found to be inversely proportional to the oxygen tension within the culture flask.

4. The optimum oxygen and carbon dioxide tensions for growth and toxin accumulation were found to be supplied by an atmosphere containing from 15 to 20 per cent oxygen and from 5 to 10 per cent carbon dioxide. In the presence of such an atmosphere uniform maximum toxin production occurred in a given medium, and no decrease in toxicity took place on prolonged incubation.

5. The reaction of cultures of *C. diphtheriae* which were grown in meat-infusion broth prepared with Difco-Proteose Peptone was maintained at about pH 8.0, pH 7.8 and pH 7.5 by aeration with atmospheres containing 5, 10 and 20 per cent carbon dioxide, respectively.

6. The ammonia content of all cultures showed a sharp increase during the period of maximum growth. In the case of the cultures grown under ordinary atmospheric conditions, or aerated with CO₂-free atmosphere, this increase was followed by a gradual decline, as compared to a continuous increase throughout the entire twenty-day incubation period in cultures grown under an increased carbon dioxide tension.

7. The amino nitrogen increase in cultures grown in broth prepared with Difco-Bacto Peptone, was much less marked than in cultures grown in broth prepared with Difco-Bacto Peptone, presumably due to a dearth of protein derivatives in Difco-Bacto Peptone which are capable of being broken down into amino acids by the peptolytic enzymes of *C. diphtheriae*.

8. The proteose fractions of certain commercial peptones were found to be necessary for toxin formation for other reasons than their buffering action on decreasing hydrogen ion concentration of the culture medium.

9. Increased carbon dioxide tension was found to prevent the usual rapid destruction of the bacterial cells after the period of maximum growth.

10. The general conclusion may be drawn from the data presented, that carbon dioxide plays an important rôle in the growth and toxin production of *C. diphtheriae*, mainly by:

a. Acting either as a catalyst or food in stimulating growth and toxin formation.

b. Controlling the reaction of the culture medium during growth.

c. Preventing the destruction of the toxin once it is formed, by a mechanism which is as yet little or not at all understood.

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