Of the three preparations examined, Triphal shows a much lower toxicity, greater stability in powder form and also in solution. Solutions of gold and sodium thiosulfate decompose readily, yielding a white precipitate and giving a marked odor of hydrogen sulfide.

This is a preliminary report.

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Carbon Dioxide and Bacterial Toxin Production: Preliminary Report on Diphtheria Toxin.

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Considerable difficulty is still being experienced in obtaining uniformly potent toxins, even under the best of known conditions. The purpose of this investigation has been to determine whether carbon dioxide bears any definite relationship to toxin production.

The commonly used medium containing meat infusion, 2 per cent Difco Proteose Peptone and 0.5 per cent NaCl was employed. The 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 pressure for fifteen minutes.

Immediately after cooling each flask was inoculated with 1 cc. of a 24 hour broth culture of *C. diphtheriae* (Park No. 8).

The inoculated flasks were divided into 4 sets, 4 flasks in each set, and incubated under the following conditions: 1 was incubated under ordinary conditions. 2 was aerated with air. 3 was aerated with carbon dioxide free air. 4 was aerated with an atmosphere containing 3 per cent carbon dioxide.

All aerated flasks were aerated at the rate of 100 cc. per hour, and incubation was at 37° C.

One flask was removed from each set at intervals of three, five, eight and 20 days from the time of inoculation, and the following

determinations made on each culture: (1) pH at time of harvesting, (2) mg. of ammonia per 100 cc. of culture, (3) amount of sediment per 100 cc. of culture (estimated by centrifuging an aliquot portion in a calibrated centrifuge tube), (4) number of viable organisms per cc., as determined by the plating method, and (5) the toxicity, which was determined by the intracutaneous method originated by Römer and Sames[6] and later developed and applied by Glenny and Allen.[4] The results were recorded in Ln/500 doses, a dose in this case being the minimum amount of toxin which when injected intracutaneously together with 1/500 of a unit of diphtheria antitoxin causes a small area of necrosis distinguishable five days after the injection.

The results of the first experiment are recorded in Table I.

TABLE I.

Culture.	No. of days incubation	Ln/500 dose	pH when harvested	Increase in mg. NH_3-N per 100 cc. culture	Sediment per 100 cc. culture	No. of viable org. per cc. of culture
Set No. 1. (Incubated under ordinary conditions)	3	>.01	8.6	20.8	1.1 cc.	250,000,000
	5	.003	8.8	19.2	1.2 cc.	325,000
	8	.0018	8.9	16.3	1.1 cc.	2,800
	20	.005	8.9	6.4	.8 cc.	60,000
Set No. 2. (Aerated with normal air)	3	>.01	8.4	20.7	1.2 cc.	63,000,000
	5	>.01	9.0	22.0	1.2 cc.	26,000
	8	.004	8.9	21.0	1.1 cc.	3,000
	20	.008	9.0	19.0	1.1 cc.	8,000
Set No. 3. (Aerated with CO_2-free air)	3	>.01	8.4	21.7	1.2 cc.	360,000,000
	5	.0025	8.8	22.0	1.2 cc.	257,000
	8	.0025	9.0	21.0	1.0 cc.	2,000
	20	.005	9.0	20.0	1.0 cc.	1,000
Set No. 4. (Aerated with air plus 3% CO_2)	3	>.01	7.8	22.0	1.2 cc.	315,000,000
	5	.0008	7.8	23.7	1.2 cc.	227,000,000
	8	.0008	8.0	24.1	1.2 cc.	95,000,000
	20	.001	8.0	24.7	1.2 cc.	13,000,000

Results with Difco-Proteose Peptone.
> = Greater than.

All flasks were kept tightly stoppered after removal from the incubator, to prevent the escape of carbon dioxide. The amino acid content of each culture was determined by means of the Sörenson Formol Titration, but no marked differences were obtained between the aerated cultures and those which were not aerated. In both cases there was a slight drop in amino acid content during the first few days of incubation, which was followed by an increase. Surface tension determinations were made on each culture, by means

of the DuNöuy Tensiometer. No marked changes in surface tension resulted in any of the cultures.

The ammonia content of all cultures showed a sharp increase during the first three days of incubation. In the case of the cultures grown under ordinary conditions this increase was followed by a gradual decline, as compared to a continuous increase in the flasks aerated with 3 per cent carbon dioxide. Apparently the ammonia content of cultures incubated under carbon dioxide is an indication of the pronounced peptolytic activity of the diphtheria organism.

A number of investigators, including Kligler[5] and Bunker,[3] have suggested that the need of "peptones" of high proteose content in toxin production is possibly due to their buffering action. By aerating the culture flasks with 3 per cent carbon dioxide it is possible to control the reaction of the medium, as shown in Table I.

Experiment No. 1 was repeated, using Difco-Bacto Peptone, which is known to be relatively low in proteose content, in place of Difco-Proteose Peptone. The results are given in Table II.

TABLE II.

Culture	No. of days incubation	Ln $\frac{}{500}$ dose	pH when harvested	Increase in mg. NH_3-N per 100 cc. culture	Sediment per 100 cc. culture	No. of viable orgs. per cc. culture
Ordinary conditions	8	>.01	9.0	12.6	.8 cc.	473,000
Aerated with air	8	>.01	9.0	13.0	.8 cc.	5,000
Aerated with air plus 3% CO_2	8	>.01	7.8	17.3	1.0 cc.	25,000,000

Results with Difco-Bacto Peptone.

These results show that little, if any, toxin was obtained with this peptone, even though the reaction was kept close to the initial pH. It would seem that the proteose fractions of "peptones" are necessary for toxin production for other reasons than their buffering action.

Apt and Loiseau,[2] Apt,[1] and Wolf[7] have found that the diphtheria bacillus, when grown in sugar-free peptone meat infusion broth, produces considerable amounts of carbon dioxide by the destruction of amino-acids, enough to have a marked effect on the reaction of the culture.

The results presented in this paper show that the carbon dioxide content of diphtheria cultures has considerable influence on the amount of toxin present when the cultures are harvested. In cases

where large numbers of cultures are grown in an incubator with poor ventilation, enough carbon dioxide may accumulate to prevent the deterioration of the toxin, once it is formed. Where few cultures are grown in incubators with better ventilation, the carbon dioxide readily diffuses out of the medium, resulting in a more rapid rise in pH and more rapid deterioration of the toxin. The obtaining of a more potent toxin and a lower pH where flasks containing a liter or more of culture are used, as compared with small flasks containing 100 cubic centimeters or less, may be explained as a rule in a similar way.

CONCLUSIONS.

1. Carbon dioxide plays an important rôle in toxin production, by preventing the destruction of the toxin, once it is formed. This is probably done by regulating the reaction of the medium.

2. It is possible to control the reaction of *C. diphtheriae cultures* of an initial pH 7.4 to 7.6 by passing an atmosphere containing from 3 to 5 per cent CO_2 over them. By this procedure an abundant supply of oxygen is maintained.

3. Increased CO_2 tension also prevents the rapid destruction of the organisms present after the period of maximum growth, probably by controlling the reaction of the medium.

4. "Peptones" high in proteose content are apparently necessary for toxin production for other reasons than their buffering action.

5. More abundant growth was obtained with Difco-Proteose Peptone, either with or without increased carbon dioxide tension, than with Difco-Bacto Peptone.

6. No appreciable amounts of toxin were found during the period of most active growth, which is the period of decrease in amino acid supply. On the other hand, it seems to be formed after the period of maximum growth, and during time of increase of amino acid content.

7. These tests should help to explain the irregular results obtained in the production of diphtheria toxin when cultures of diphtheria organisms are grown under ordinary conditions.

This is a preliminary report.

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