FURTHER STUDIES UPON THE EFFECT OF VARIOUS CARBOHYDRATES ON PRODUCTION OF DIPHTHERIA TOXIN WITH SPECIAL REFERENCE TO ITS FLOCCULATING TITER AND FINAL pH

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INTRODUCTION

As early as 1896 Park and Williams (1896) recommended the use of glucose in small quantities as an aid in the production of toxic filtrates from *C. diphtheriae*. In contrast to Spronck (1895) they found, moreover, that the muscle sugar present in the infusion broth was not harmful to toxin production. T. Smith (1899) a short time afterwards, following a very complete study on the relation of glucose to the production of diphtheria toxin, reported that glucose added in small amounts to sugar-free meat infusion broth considerably enhanced the production of diphtheria toxin. Since this early work apparently little attention has been paid to the use of glucose in toxin production until the work of Wadsworth and Wheeler (1928). These authors found that glucose is essential for growth and toxin production in meat-infusion sugar-free peptone medium. Locke and Mann (1928) and Ramon (1929) have also only recently reported the use of glucose in the production of high-titered diphtheria toxin. Glucose in 0.2 per cent concentration is used routinely in the production of diphtheria toxin by the Division of Laboratories and Research of the New York State Department of Health. Earlier in the year we (Hazen and Heller) (1931) published a brief report of studies in which glucose and maltose had been used to enrich culture media for growth of *C. diphtheriae*. We found that the addition of either

sugar to the culture medium definitely increased the Lf[1] unitage of the filtrates and that the single addition of a combination of the two sugars yielded filtrates of still higher potency within a period of only five days.

The present work is a further study of the effect of glucose and maltose on the production of diphtheria toxin together with a study of other carbohydrates fermentable by *C. diphtheriae*, such as dextrin and glycerol.[2] At the same time we have endeavored to correlate as far as possible the final pH of the filtrates with the Lf titer.

TECHNIQUE

The medium used in these experiments was made of bob veal infusion to which was added 2 per cent proteose peptone. Especial attention was paid to the quality and freshness of the veal as well as to the sterilization of the medium. The final pH of the medium was 7.8. The sugars we made up in 20 per cent solutions. Formerly they were heated in the Arnold for twenty minutes for three successive days; more recently it has been found more efficacious to sterilize the solutions by filtration through a Berkefeld candle. Only chemically pure sugars were used in this work. The Park-Williams No. 8 strain which was used throughout the work was maintained by transfer twice daily in the veal infusion broth.

The broth was inoculated with one large loopful of an actively growing pellicle. The cultures were grown in 300 cc. quantities in 1-liter flasks. The flasks (diagram No. 1) were specially designed so that frequent pH titrations and flocculation tests, if desired, could be carried out on the same specimen without disturbing the pellicle by manipulation of the flask.

Determinations of the M.L.D. were made on only a few of the

[1] The term is used in accordance with Glenny and Wallace's definition indicating "that amount of toxin (corresponding to one unit of a certain antitoxin) in that mixture which flocculates most rapidly when a series of mixtures of that toxin and antitoxin are set up in varying proportions and observed under constant conditions" (Topley and Wilson).

[2] Classed with the carbohydrates only because it is fermentable by *C. diphtheriae*.

filtrates. The Ramon flocculation test was carried out in the routine manner on the various filtrates.

EXPERIMENTAL WORK

The various carbohydrates (glucose, maltose, dextrin, and glycerol) were utilized as follows:

1. Small amounts of the single carbohydrate were added to the growing culture once or twice daily for a period of forty-eight

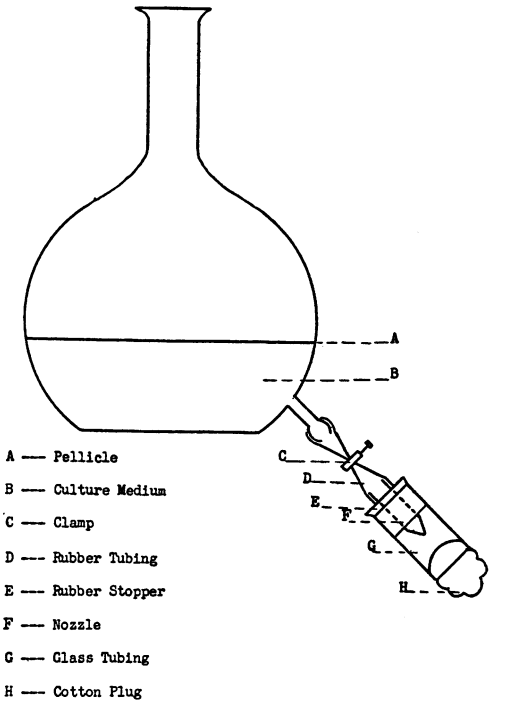


DIAGRAM 1. SPECIAL FLASK

to seventy-two hours, each addition of the sugar being preceded by pH titrations and adjustment of the culture fluid to pH 8.0. Control flasks with the same sugar concentrations, remaining unadjusted, were carried along at the same time.

2. The carbohydrate (glucose, maltose, dextrin, glycerol) was added to the medium before planting the pellicle with daily titration of the culture fluid.

3. A given amount of two carbohydrates in combination was added to the broth before planting the pellicle with daily adjustment of the culture fluid to pH 8.0. Control flasks of similar composition but unadjusted pH were run at the same time.

I. EFFECT OF FRACTIONAL ADDITION OF VARIOUS CARBOHYDRATES ON THE Lf TITER OF TOXIC FILTRATES OF C. DIPHTHERIAE

Semidaily additions of glucose in 0.15 per cent concentration to the growing filtrates with and without pH adjustment

Sterile 20 per cent glucose solution was added to six 1-liter flasks, containing 300 cc. of broth so as to yield a final concentration of 0.15 per cent. After preliminary incubation for sterility these flasks were divided into two groups, I and II. The first group, I, consisted of 3 flasks numbered 1, 2 and 3; the flasks in the second group were numbered 4, 5 and 6. Each of the six flasks was inoculated with one large loopful of a young, actively growing pellicle incubated at 37.5°C. Sixteen hours later 30 cc. of filtrate was withdrawn from flasks 1 and 4 for pH titration and Lf determinations. Flasks 1, 2 and 3 (group I) were then adjusted to a pH of 8.0 by adding the required amount of 50 per cent NaOH solution, the flasks in group II remaining unadjusted. Glucose solution was then added again to all six flasks to obtain 0.15 per cent concentration. In order to avoid too drastic a reduction of volume the experiment was arranged in such a manner that no more than 2 samples were drawn from the same flask during the whole test. Eight hours after the second addition of the sugar, flasks 1 and 4 were removed from the incubator and pH titrations and Lf determinations again made on the filtrates. The remaining flasks of group I were brought to pH 8.0, while the sugar concentrations, in all four flasks (2, 3, 5 and 6) were again reëstablished to 0.15 per cent. Sixteen hours later, specimens were again obtained from flasks 2 and 5 for pH titration and flocculation. Adjustment of pH of flasks 2 and 3 (group I) and addition of sugar to the four flasks, as previously described were again carried out. Eight hours later, flasks 2 and 5 were

removed from the incubator and titration of pH and flocculation tests were made on their filtrates. The two last flasks, numbers 3 and 6, were treated as in the beginning. Sixteen hours later, titration of the pH and flocculation tests on samples from these flasks were made in the usual manner followed by adjustment of the pH of flask 3 and the final addition of glucose in a concentration of 0.15 per cent to the two flasks (3 and 6). Eight hours later, i.e., seventy-two hours after the beginning of the experiment, the last two flasks were removed from the incubator for a final pH titration, and flocculation of the filtrates.

The results of the pH titrations and the Lf values of the two groups of flasks are given in chart 1.

As can be seen from chart 1, there was a rapid and continued increase in toxin production during the seventy-two hours' incubation in the flasks (1 to 3) in which there had been an adjustment of the hydrogen-ion concentration. The final value obtained was 15 Lf per cubic centimeter with a pH of 8.7. In the flasks (4 to 6) receiving the same amount of glucose but remaining unadjusted there was a much slower accumulation of toxin so that at the end of the seventy-two hours only one-half the Lf value of the adjusted filtrate was reached. However, an Lf of 8.7 per cubic centimeter within seventy-two hours is not insignificant with a pH as low as 5.6. The control broth alone at this time yielded only 9 Lf per cubic centimeter with a pH of 8.2.

It is interesting to note here that the flocculating time of the unadjusted filtrate was occasionally 5 times as long as that of the adjusted filtrates.

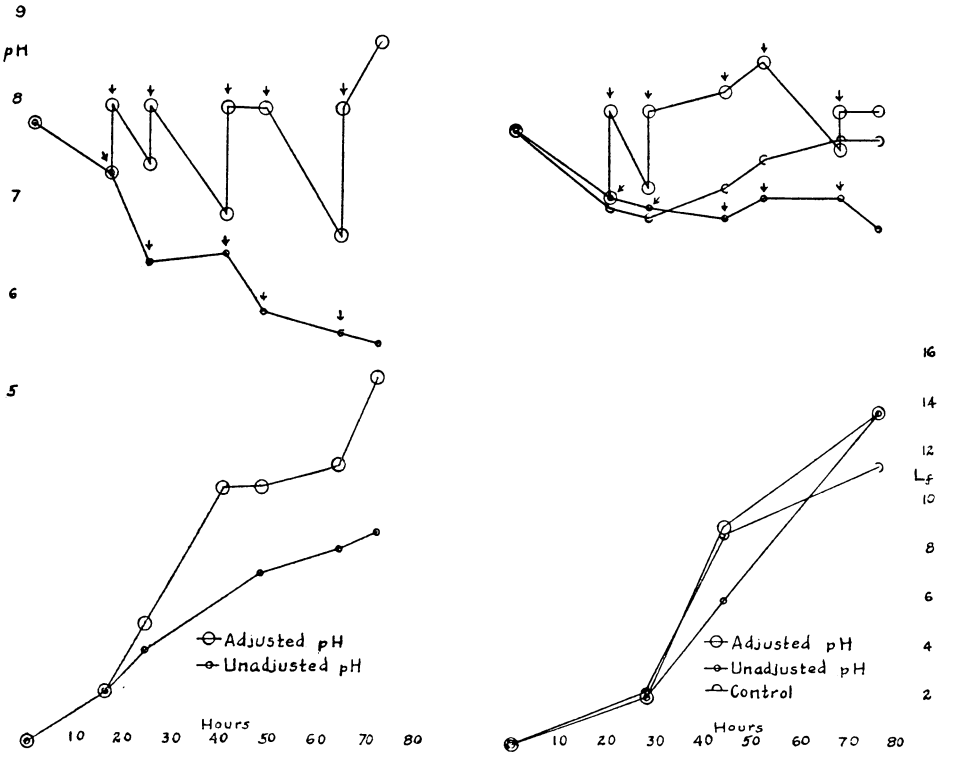
Semidaily additions of maltose in 0.15 per cent concentration to the growing culture filtrates with and without pH adjustment

The technique for this experiment is essentially the same as given above in detail except that maltose was substituted for glucose.

The results of the pH titrations and Lf determinations on the different filtrates are given in chart 2.

The Lf graphs of chart 2 show that there is an enhancement in toxin production when small amounts of maltose are frequently

added to the growing cultures of *C. diphtheriae*. The chart would also seem to indicate that there is no advantage to be gained from an adjustment of the hydrogen-ion concentration of the culture medium at the time of adding the maltose, as was found



Arrow indicates addition of carbohydrate

CHART 1 CHART 2

CHART 1. EFFECT OF REPEATED ADDITIONS OF GLUCOSE (0.15 PER CENT CONCENTRATION) ON Lf AND pH OF DIPHTHERIA TOXIN

CHART 2. EFFECT OF REPEATED ADDITIONS OF MALTOSE (0.15 PER CENT CONCENTRATION) ON Lf AND pH OF DIPHTHERIA TOXIN

necessary in the experiment with glucose. While the final pH of the unadjusted filtrate was 6.8, of the adjusted filtrate 8.0, and the control 7.7, the final Lf of both of the maltose filtrates was 13.7 per cubic centimeter contrasted with an Lf of 11.5 per cubic centimeter for the control.

Semidaily additions of dextrin in 0.15 per cent concentration to the growing culture filtrates with and without pH adjustment

The experimental procedure here is the same as that given for the first experiment except that dextrin was substituted for glucose.

The results obtained from this experiment are given in chart 3. The Lf graphs in chart 3 show that frequent additions of dextrin to growing filtrates with adjusted or unadjusted pH do not enhance toxin production materially, at least not during a period of seventy-two hours. The pH curve of the unadjusted filtrate for the first forty-eight hours suggests that dextrin is utilized only very slowly by *C. diphtheriae*. The final pH of the adjusted filtrate was 8.3, that of the unadjusted 6.5, while that of the control broth was 8.1. The Lf titers of the three filtrates differed only slightly, with an Lf of 9.5 per cubic centimeter for the unadjusted filtrate and the control and 10.5 Lf per cubic centimeter for the adjusted filtrate.

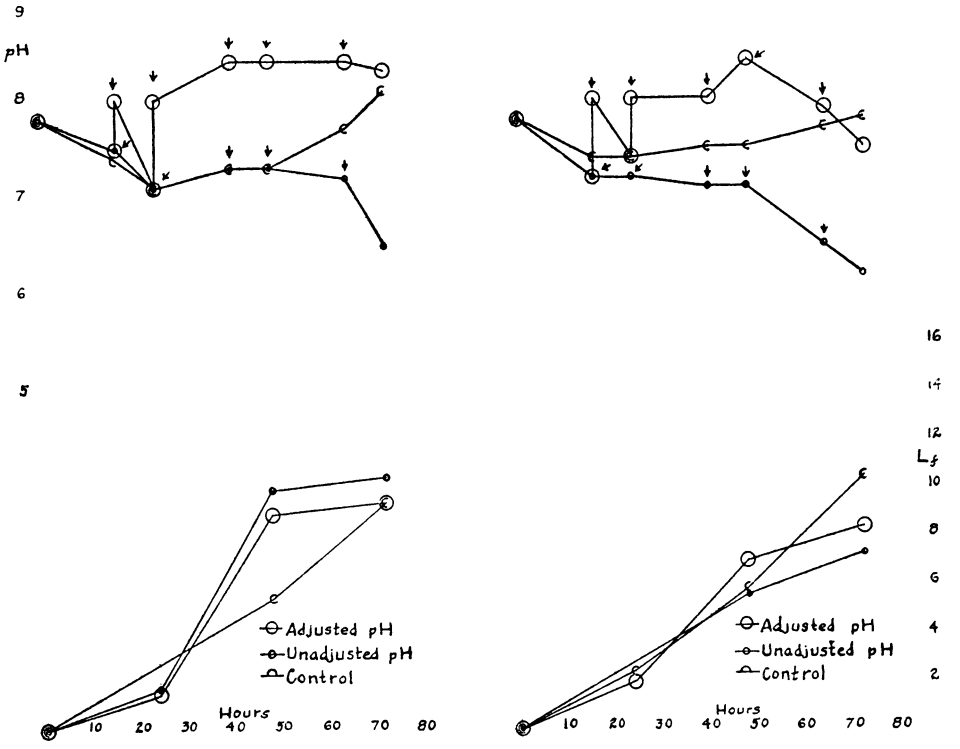
Semidaily additions of glycerol in 0.15 per cent concentration to the growing culture filtrates with and without pH adjustment

The details of such an experiment have been fully described in the glucose protocol.

Chart 4 would suggest that frequent additions of glycerol actually inhibited toxin production. The adjusted filtrate showed a final Lf titer of 8.4 per cubic centimeter; the unadjusted filtrate 7.3 while the broth control had a final titer of 10.5 Lf per cubic centimeter. The pH graphs of the glycerol filtrates show a final pH of 7.5 for the adjusted filtrates, 6.2 for the unadjusted filtrate and a final pH of 7.7 for the control broth.

In summing up our observations, it would seem that semidaily additions of glucose to growing diphtheria cultures considerably enhance the Lf titer of the toxin, provided the pH of the filtrates is adjusted at the time of adding the sugar. Fractional additions of maltose to the growing cultures were likewise found to increase the flocculating titer of the filtrates. However, adjustment of the pH of the culture fluid at the time of adding maltose was found to be unnecessary. Frequent additions of small amounts of dextrin to the cultures, with or without adjustment of pH, were

not found to affect the Lf titer of the filtrates. Finally, frequent additions of glycerol to the growing cultures were actually deleterious to the production of toxin.



Arrow indicates addition of carbohydrate

CHART 3 CHART 4

CHART 3. EFFECT OF REPEATED ADDITIONS OF DEXTRIN (0.15 PER CENT CONCENTRATION) ON Lf AND pH OF DIPHTHERIA TOXIN

CHART 4. EFFECT OF REPEATED ADDITIONS OF GLYCERINE (0.15 PER CENT CONCENTRATION) ON Lf AND pH OF DIPHTHERIA TOXIN

II. EFFECT OF A SINGLE ADDITION OF GLUCOSE, MALTOSE, DEXTRIN OR GLYCEROL TO GROWING CULTURES OF C. DIPHTHERIAE ON THE Lf TITER OF THE TOXIN

Effect of 1 per cent sugar concentration (glucose, maltose)

Eight 1-liter flasks, each containing 300 cc. of broth, were divided into two sets of four each. To one-half of the flasks 1

per cent glucose was added, the other half receiving 1 per cent maltose. The 8 flasks, together with a plain broth control flask were then inoculated and, after twenty-four hours' incubation, a pH titration as well as an Lf titration was made on all filtrates. Two flasks from each series were then adjusted to a pH of 8.0, this procedure being repeated for five consecutive days. The results of the daily titrations are given in chart 5.

The results of this experiment show that a single addition of 1 per cent glucose inhibits toxin production in so far as can be determined by the flocculation test, for at no time were we able to obtain flocculation with any of these glucose filtrates, adjusted or unadjusted. Growth in the flasks containing glucose was poor at the end of twenty-four hours. Although the pH never fell below 6.0 the rapid accumulation of acid within the first twenty-four hours of growth was apparently sufficient to handicap the organisms for further toxin production, to such an extent that later adjustment of the pH to 8.0 had no effect.

Quite opposite results were obtained with the maltose. With both adjusted and unadjusted filtrates, there was a continuous and abundant production of toxin, the final Lf attaining 17.5 per cubic centimeter while with the control broth only an Lf of 9 per cubic centimeter was obtained. Here again, adjustment of the pH of the culture was of no value. At no time did the pH of the unadjusted filtrate fall below 6.6. It is interesting to note that these filtrates were found to contain between 1000 to 1200 M.L.D.'S per cubic centimeter when tested subcutaneously on guinea pigs of 250 grams. Further experiments with maltose demonstrated that as much as 3 per cent could be added at one time to the culture fluid without producing a deleterious effect.

Effect of 0.45 per cent carbohydrate concentration

Twelve flasks containing 300 cc. each of broth were divided into four sets of three flasks each. To each set of flasks there was added 0.45 per cent concentration of glucose, maltose, dextrin and glycerol respectively. The entire series, together with a control flask of plain broth, was inoculated with a fresh pellicle of *C. diphtheriae* and daily titrations of the pH and Lf determina-

tions of the different filtrates were carried out for five days. The results obtained in this experiment are given in chart 6.

As may be seen from chart 6 a single addition of 0.45 per cent concentration of carbohydrate to the culture fluid at the time of

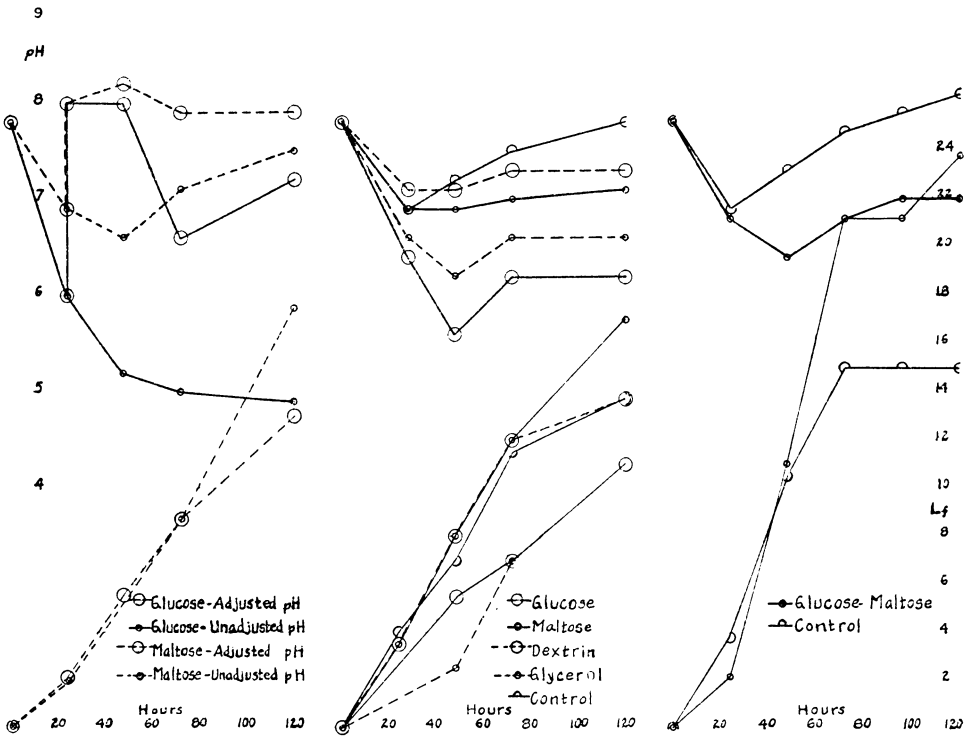


CHART 5 CHART 6 CHART 7

CHART 5. EFFECT OF A SINGLE ADDITION OF CARBOHYDRATE (1 PER CENT CONCENTRATION) ON Lf AND pH OF DIPHTHERIA TOXIN

CHART 6. EFFECT OF A SINGLE ADDITION OF CARBOHYDRATE (0.45 PER CENT CONCENTRATION) ON Lf AND pH OF DIPHTHERIA TOXIN

CHART 7. EFFECT OF A SINGLE ADDITION OF GLUCOSE (0.15 PER CENT CONCENTRATION) AND MALTOSE (0.3 PER CENT CONCENTRATION) ON Lf and pH OF DIPHTHERIA TOXIN

planting the pellicles is of doubtful value with the majority of the carbohydrates tested. Only the filtrates containing maltose in 0.45 per cent concentration showed a higher titered toxin than

the control in five days. While the filtrates containing dextrin in the same concentration had an Lf titer equal to that of the control, the Lf titer of the filtrates containing 0.45 glucose concentration was less than that of the control. We were unable to obtain a flocculation phenomenon with the filtrate containing 0.45 per cent concentration of glycerol after the third day of incubation when the observed Lf titer was 7 per cubic centimeter. The marked inhibition in toxin production in the glycerol-containing medium can hardly be explained on the basis of excessive acidity for the lowest pH reached during the five days of incubation was 6.2.

TABLE 1

Effect of a single addition of carbohydrate (0.15 per cent concentration) on Lf and pH of Diphtheria toxin

CONCENTRATION OF CARBOHYDRATE IN CULTURE MEDIUM	FINAL pH (5 DAYS' INCUBATION)	Lf PER CUBIC CENTIMETER (5 DAYS' INCUBATION)
0.15 per cent glucose	7.7	9.0
0.15 per cent maltose	7.8	7.5
0.15 per cent dextrin	7.7	5.0
0.15 per cent glycerol	8.0	8.5
Plain broth control	7.0	6.0

Effect of 0.15 per cent carbohydrate concentration

The results of the final titration of the hydrogen-ion concentration of the filtrates and the final Lf values for the different filtrates are given in table 1.

It will be seen from table 1 that a single addition of maltose or glycerol in 0.15 per cent concentration to the medium at the time of planting the pellicles increased the yield of toxin within five days somewhat over that of the control, while dextrin in the same amount apparently had no effect on toxin production. Glucose on the other hand in similar amounts enhanced the Lf titer of the filtrates considerably. While a small addition of glucose to the culture fluid appears to favor toxin production more than maltose

in equivalent amounts, this is not true for larger additions of the two sugars.

Addition of a given amount of two carbohydrates in combination to the culture fluid

We next attempted to determine the effect on toxin production of combining the two sugars. Since adjustment of the pH of the culture filtrates is laborious as well as impractical for routine work, we thought that by adding glucose and maltose simultaneously in varying amounts it might be possible to obtain a combination which would not only augment the yield of toxin but at the same time prevent the drop in pH which accompanies the fermentation of glucose by *C. diphtheriae*.

The final results obtained from the single addition of the combined sugars are shown in table 2.

TABLE 2

Effect of a given amount of two carbohydrates in combination on Lf and pH of diphtheria toxin

FLASKS	CONCENTRATION OF GLUCOSE IN MEDIUM	CONCENTRATION OF MALTOSE IN MEDIUM	FINAL pH (5 DAYS)	M.L.D.	Lf PER CUBIC CENTIMETER
	per cent	*per cent*			
Control			7.7	770	9.6
A	0.15	0.15	7.4	1,400	17+
B	0.15	0.3	7.1	2,100	26.3
C	0.3	0.15	6.9	1,400	17+
C	0.3	0.3	6.4	1,260	15.7

It will be noted from table 2 that in every flask to which the two carbohydrates were added there was an increase in the Lf titer of the filtrates when compared with that of the plain broth control. Since a combination of 0.15 per cent glucose and 0.3 per cent maltose yielded filtrates of the highest Lf value, with a final pH of 7.1 at the end of five days, we used the two sugars in these amounts in further experiments and obtained uniformly most satisfactory results. Chart 7 shows a typical experiment.

Our experience in the past year with a variety of toxins produced by various carbohydrates has in every instance indicated the best results with the use of this combination. During the winter months, when diphtheria toxin is more difficult to produce

in high titer we obtained with this method within five days filtrates of 15, 17 and 21 Lf per cubic centimeter. Povitsky (1931) reports that she was unable to obtain in a volume of 600 cc. a higher titered toxin by addition of the combined sugars (glucose and maltose) than was obtained by the routine method (0.15 per cent concentration of glucose) and that the pH of those filtrates made them impractical for toxoid purposes. We have been able to produce toxoids from such filtrates (pH 7.1) within the usual time limit for toxoid production. Such toxoids, moreover, flocculated within twenty-five to forty-five minutes with the same antitoxin which we used with the original toxin. Dr. Florence Stone in this Department has obtained with this method in a volume of 100 cc. within five days filtrates of 30 or more Lf per cubic centimeter. With the proper adjustment of the initial pH of the medium and sterilization of the sugar by Berkefeld filtration it should be possible to obtain satisfactory filtrates with a volume of 600 cc. by this method.

SUMMARY

Studies on the daily changes in pH and toxin production in media containing glucose, maltose, dextrin and glycerol after inoculation with *C. diphtheriae* have yielded the following results:

1. Glucose added twice daily in concentration of 0.15 per cent to the growing cultures during the period of seventy-two hours' incubation definitely increased the Lf titer of the filtrates, provided the medium was alkalinized at the time the sugar was added. Similar additions of maltose in 0.15 per cent concentration likewise increased the Lf titer of the filtrates without resorting to pH adjustment. Dextrin or glycerol under similar experimental conditions failed to increase the yield in Lf units per cubic centimeter.

2. Glucose added only once to the culture broth at time of inoculation in 0.15 per cent concentration stimulated a more abundant toxin production within five days than was obtained with glucose free broth. A fairly potent toxin was also harvested with glucose added in 0.45 per cent concentration to the culture fluid at time of inoculation, but the titer did not equal that of

the control broth. Larger amounts of glucose inhibited toxin production. Smaller amounts of maltose added at the time of seeding likewise increased the Lf unitage of diphtheria toxin, excellent and equally good results being obtained with 0.45 and 1 per cent concentrations of this sugar. A single addition of dextrin in the amounts stated above did not increase the Lf value while glycerol added in 0.15 per cent concentration definitely enhanced toxin production. Lower values were obtained with larger quantities of glycerol.

3. With a combination of glucose and maltose (0.15 and 0.3 per cent concentration) added to the culture fluid at time of inoculation, filtrates containing as high as 26.3 Lf per cubic centimeter were obtained within five days. This combination of sugars proved to be more efficacious than any other method tried.

The final reactions at which highly potent filtrates were harvested were not found to lie within the commonly accepted range of pH 7.8 to 8.25 (Bunker (1919)). The strongest toxins had a final pH of 7.1. Daily titrations of pH moreover showed that the reactions never approached the critical zone laid down by Bunker. This author found potent toxins only in cultures with a final pH of 7.8 to 8.20. Our observations therefore agree with those of Hartley and Hartley (1922) who also reported high toxins with variations in pH from 7.5 to 8.9. Since our work was based on toxin production with a comparatively small volume (300 cc.) we have no way of telling whether different results may be obtained with larger quantities.

CONCLUSIONS

1. Carbohydrates are essential for the production of toxic filtrates of *C. diphtheriae* possessing highest potency.

2. Glucose in small amounts is definitely superior to maltose, dextrin or glycerol as an aid to toxin production.

3. Larger amounts of maltose than it is possible to use in the case of glucose without adjustment of pH increase the Lf titer of the filtrates of *C. diphtheriae*.

4. Dextrin in the amounts tested has no demonstrable effect on toxin production.

5. Glycerol in 0.15 per cent concentration increased toxin production while larger quantities were inhibitory.

6. Filtrates of the highest Lf were obtained with a combination of glucose in 0.15 per cent concentration and maltose in 0.3 per cent concentration.

7. The final pH of the filtrate does not necessarily seem to be an infallible index as to the best time of harvesting the toxin. It would seem therefore that the toxicity of a given filtrate is largely independent of the particular final pH reaction at the end of incubation.

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