Increased Toxin Titres Obtained by the Addition of Selected Carbon Sources to Shaken Cultures of *Corynebacterium diphtheriae*

By E. D. SINGER, LEA GRUNDMAN AND FANY CROITORU

*Department of Bacteriology and Immunology,
The Rafa Research Laboratories, Jerusalem, Israel*

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SUMMARY

The rate of utilization of maltose in shaken cultures of a strain of *Corynebacterium diphtheriae* Park Williams 8 was much higher than that of the N source. Considerable gain in toxin yields was achieved when maltose or some intermediates of its metabolism (glucose, glycerophosphate, lactate, acetate) were added to the cultures between 20 and 32 hr of incubation. When, however, the initial concentration of these carbon sources in the medium was increased above the usual value (25 mg./ml.) a decrease in toxin formation resulted. The carbon intermediates were active at concentrations up to 15 times lower than that of maltose. Their action on the toxin titre was immediate, as contrasted to the lag of 3-4 hr which followed the addition of maltose.

INTRODUCTION

The introduction of submerged cultures (Linggood & Fenton, 1947; Linggood *et al.* 1955) instead of surface cultures for large-scale production of diphtheria toxin yielded not only more toxin but primarily a much greater bacterial mass. This fact led us to the assumption that some of the nutrients which were present in excess in the surface cultures might in submerged cultures reach suboptimal concentrations that limited the further increase in toxin titres. Preliminary studies have shown that the rate of utilization of the carbon source (maltose) was much higher than that of the nitrogen source. The present paper deals therefore with the results of a study aimed to achieve higher toxin yields by adding to the medium maltose or some other carbon sources which are intermediates of maltose metabolism.

METHODS

Organism. A culture of the Park Williams no. 8 strain of *Corynebacterium diphtheriae* (CN2000 from the National Institute of Public Health, Utrecht, Netherlands, by the courtesy of Dr A. Tasman) was used throughout.

Medium. The organisms were cultivated on a medium based on papain digest of beef muscle as described by Linggood *et al.* (1947) with the exception that our routine medium contained 2.5% (w/v) maltose. The medium was sterilized by filtration through Seitz filters and steamed for 20 min.

Toxin. Toxin was estimated by the flocculation method (Ramon, 1922) with a

purified antitoxin calibrated against the standard antitoxin for flocculation of the National Institutes of Health, Bethesda, U.S.A.

Growth. The extinction of washed and diluted samples of bacterial culture was determined with a Beckman B spectrophotometer at 540 m μ .

Maltose. Maltose utilization was estimated in the supernatant fluid of centrifuged cultures by the method of Somogyi (1945).

Amino-nitrogen. The amino-nitrogen content of the supernatant fluids of centrifuged cultures was estimated by the method of Pope & Stevens (1939).

Total nitrogen. The method of Ma & Zuazaga (1942) was used to estimate total N in the supernatant fluids of centrifuged cultures.

Toxin production. Toxin was produced in 1 l. Erlenmeyer flasks, each containing 100 ml. medium. Each flask was inoculated with 5 ml. of an 18–24 hr. shaken culture and mounted on a reciprocating shaker (120 strokes/min.) which could accommodate 56 bottles, and shaken for 48 hr in an incubator at 36–37°. In kinetic studies, samples of two to seven flasks were removed at intervals, examined microscopically to test the purity of the cultures, pooled and submitted to the tests described.

RESULTS

Influence of added carbon sources on the toxin titres

Preliminary experiments have shown that the rate of maltose utilization in shaken cultures of *Corynebacterium diphtheriae* was so high that in many cases it disappeared almost completely from the medium within 48 hr (duration of the process), whereas a large excess of the N source always remained. In the 18 experiments given in Table 1 the percentage of total nitrogen utilized varied from 1.1 to 31.2 %, average 20.95 %. For amino-N the values were 3.7–54.4 %, average 30.8 %, while for the maltose values were 29.6–99 %, average 82.1 %.

It was assumed that because of the high rate and extent of maltose utilization, the concentration of this nutrient in the medium might become (after decreasing below a certain value) suboptimal for toxin formation. When, however, the initial concentration of maltose in the medium was increased above the usual value (25 mg./ml.), an unexpected decrease (of about 25 %) in toxin formation resulted. The timing of maltose addition was therefore changed, and it was added to the cultures at a phase when a considerable amount of maltose was already used up and the rate of toxin formation was high. Indeed such a treatment resulted in considerable improvement in toxin yields. Concentrations of added maltose ranging from 1 to 25 mg./ml. were investigated. The lowest concentration which still caused a limited but reproducible increase in toxin titre was 5 mg./ml. On an average the best results were obtained when maltose 15 mg./ml. were added to the cultures any time between 20 and 32 hr of incubation.

To see whether the effect of maltose on the toxin titres was due specifically to maltose or to the elimination of some partial deficiency in a readily available carbon source at a critical period, the same treatment was repeated with some intermediates of maltose metabolism; glucose, lactate, glycerophosphate, acetate. (Lactate and acetate are ingredients of the routine culture medium.) The intermediates acted in a similar way to maltose; their addition at 0 hr caused a decrease in toxin titres, whereas when added to the growing cultures between 20 and 32 hr, they caused a comparable

rise in toxin yields. A significant difference was, however, observed: the concentrations of the intermediates which were as effective as maltose were up to 15 times less than that of maltose.

Table 1 summarizes the results of some of the experiments in which the carbon sources were added to the incubating cultures. The values given in Table 1 were determined on pooled cultures. The number of cultures pooled in each experiment varied between 2 and 10 according to the number of variations tested and according to whether or not cultures were also taken at intermediate times of incubation. The fact that in some cases only few cultures were pooled does not detract from the significance of the results obtained because this was done only in kinetic studies in which the effects were measured and verified on many samples at consecutive periods of time (see Figs. 1-3). Since within the given period, the exact time of addition was not found to be critical, only those experiments are given in Table 1 in which the additions were made at 24 hr of incubation. For convenience almost all the experiments in which the effect of only one kind of carbon source was compared with untreated controls are omitted from Table 1. In spite of this selection, only in the case of maltose and glucose 1 mg./ml. were there slight deviations from the overall average gains in titre. The overall gain as a consequence of adding maltose was 25 % instead of 23 % as in the experiments listed in Table 1, whereas in the case of glucose 1 mg./ml. the overall gain was 23 % instead of 25 % in the experiments listed in the table.

The variation in toxin yields of control cultures was considerable. This can be attributed to differences in the quality of meat used for preparing the digest and/or to variations in the degree of deferration of the different batches of media. It is also difficult to exclude slight variations in the physiological state of the inocula. Experience has shown that in course of routine production variations in titres are not infrequent and vary from laboratory to laboratory. Therefore it seems to be relevant that, following the addition of carbon sources, increased toxin titres could be obtained in each experiment regardless of the toxin yield in control cultures.

It can be seen from Table 1 that the best results were obtained with acetic acid 0.97 mg./ml. About 41 % gain in toxin yield was achieved in this case which represented about 80 Lf/ml. as compared to 36 % (about 70 Lf/ml.) in the case of glucose 2.5 mg./ml. and about 50 Lf/ml. in the case of maltose 15 mg./ml. or glucose 1 mg./ml.

The addition of maltose always caused a decrease in final pH values, which was not general in the case of the intermediates. The addition of acetic acid 0.97 mg./ml. decreased the pH value by about 1 unit at the time of addition, but in later phases of incubation even higher pH values than those of the controls were obtained. This phenomenon, as pointed out by Linggood *et al.* (1947) was probably due to the conversion of acetate to carbonate and water. Too high concentrations of added glucose or acetic acid delayed toxin formation or (in cases when values below pH 6 were obtained) even caused the destruction of the toxin formed before these additions.

Kinetics of the influence of some of the carbon sources on the rate of toxin formation

The finding that the maltose metabolism intermediates influenced the toxin titre at much lower concentrations than did maltose itself pointed to possible differences in the respective kinetics of their action on toxin formation. The rate of toxin formation immediately after the additions at 24 hr was therefore studied. Treated and control

Table 1. *Effect of maltose or of some of the intermediates added to growing shaken cultures of C. diphtheriae (CN2000) on final toxin titres and pH values*

(Lf./ml. (A) and pH (B) values at the end of the experiment (48 hr.))*

Additions at the 24th of growth hr (mg./ml.)	Exp. 77		Exp. 79		Exp. 82		Exp. 84		Exp. 85		Exp. 86		Exp. 104		Exp. 105		Exp. 107	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
None	280	8.85	340	8.6	240	7.5	280	8.75	280	8.75	260	8.8	180	7.4	240	7.6	120	7.2
Maltose	—	—	360	8.1	260	7.0	360	7.8	320	7.4	320	7.5	250	7.1	320	6.55	180	6.5
Sodium lactate																		
1	300	9.0	380	7.75	—	—	320	8.7	300	8.4	300	8.8	—	—	—	—	—	—
3	310	9.1	380	8.65	—	—	—	—	—	—	—	—	—	—	—	—	—	—
5	—	—	—	—	—	—	—	—	—	—	320	8.95	—	—	—	—	—	—
Lactic acid																		
0.88	—	—	—	—	280	7.9	300	8.55	320	8.5	300	8.8	—	—	—	—	—	—
1.76	—	—	320	8.5	—	—	—	—	—	—	—	—	—	—	—	—	—	—
2.64	—	—	—	—	270	7.9	—	—	—	—	—	—	—	—	—	—	—	—
3.52	—	—	360	8.65	—	—	—	—	—	—	—	—	—	—	—	—	—	—
4.40	—	—	—	—	—	—	—	—	—	—	320	8.95	—	—	—	—	—	—
Glycerophosphate																		
1	—	—	—	—	260	7.0	320	8.7	300	8.5	280	9.0	—	—	—	—	—	—
3	—	—	—	—	250	7.0	—	—	—	—	—	—	—	—	—	—	—	—
5	—	—	—	—	—	—	—	—	—	—	300	9.0	—	—	—	—	—	—
Glucose																		
1	—	—	—	—	—	—	—	—	—	—	—	—	200	7.3	280	7.7	220	7.35
2.5	—	—	—	—	—	—	—	—	—	—	—	—	240	7.5	260	7.9	270	7.2
5.0	—	—	—	—	—	—	—	—	—	—	—	—	nf†	5.5	160	7.0	—	—
7.5	—	—	—	—	—	—	—	—	—	—	340	8.6	nf†	5.3	80	6.4	—	—
10.0	—	—	—	—	—	—	—	—	—	—	—	—	—	—	nf†	5.25	—	—
Acetic acid																		
0.97	—	—	—	—	—	—	—	—	—	—	—	—	280	7.7	320	8.1	260	7.25
1.94	—	—	—	—	—	—	—	—	—	—	—	—	nf†	5.7	220	8.1	nf†	5.6
	Exp. 108		Exp. 109		Exp. 123		Exp. 124		Exp. 127		Exp. 130		Exp. 131		Exp. 132		Exp. 133	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
None	120	6.7	90	7.2	320	8.85	180	8.65	300	8.25	180	8.4	300	8.3	260	8.2	200	8.2
Maltose																		
15	180	6.5	180	6.82	360	8.5	220	8.5	—	—	260	7.3	320	7.0	300	7.2	225	7.2
Sodium lactate																		
1	—	—	140	6.9	—	—	—	—	—	—	—	—	—	—	—	—	—	—
3	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
5	—	—	140	8.0	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Glucose																		
1	150	7.5	—	—	—	—	220	8.6	340	8.45	290	6.7	320	8.35	300	8.1	280	8.1
2.5	—	—	220	7.5	—	—	—	—	—	—	260	7.7	320	8.2	290	8.15	280	7.85
5.0	—	—	—	—	—	—	—	—	—	—	240	7.8	250	8.0	—	—	—	—
Acetic acid																		
0.97	220	7.2	220	7.7	380	8.9	—	—	360	8.3	220	8.0	330	8.4	—	—	260	8.1
1.94	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Sodium acetate																		
1	—	—	—	—	—	—	—	—	—	—	240	8.5	320	8.5	—	—	240	8.3
2	—	—	—	—	—	—	—	—	—	—	270	8.65	350	8.5	—	—	250	8.2
3	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	260	8.25

* Values obtained from pooling several cultures (see text).

† No flocculation.

Fig. 1. Expt. 122. Effect of adding maltose 15 mg./ml. at the 24th hr of incubation to shaken cultures of *C. diphtheriae* (CN 2000). ●—●, No addition; □—□, addition of maltose 15 mg./ml.

Fig. 2. Expt. 119. Effect of adding glucose 1 mg./ml. at 0 hr or at the 24th hr of incubation to shaken cultures of *C. diphtheriae* (CN 2000). ●—●, no addition; ×—×, addition at 0 hr 1 mg./ml. glucose; ○—○, addition at 24 hr 1 mg./ml. glucose.

flasks were removed from the shaker at intervals of 1–1.5 hr and their contents examined.

The results are presented in Figures 1–3.

After adding 15 mg. maltose/ml. (Fig. 1), more than 4 hr elapsed before the titre in the treated cultures started to increase above the value in the controls. It can be

seen from the curves of reducing activity that about 13 mg. maltose/ml. were present in the medium at the time of addition and that the addition caused an increase in the rate of maltose utilization. Final maltose concentrations were identical in both treated and control cultures. The pH value decreased because of conversion of maltose to acids and remained lower than that of the controls until the end of the experiment. In general growth was promoted and more nitrogen source was used up as a consequence of the addition. There were, however, exceptions in which the curves of the growth or nitrogen sources did not follow this pattern.

Fig. 3. Expt. 127. Effect of adding acetic acid 0.97 mg./ml. at the 24th hr of incubation to shaken cultures of *C. diphtheriae* (CN 2000). ●—●, no addition; ○—○, addition of 0.97 mg./ml. acetic acid.

In Fig. 2 the effect of adding glucose 1 mg./ml. at 0 hr or at 24 hr is shown. As a consequence of the addition at 0 hr the rate of toxin formation was retarded and the pH value in the first hours of growth decreased to lower values than those of the controls.

In contrast to what was observed on adding maltose at the 24 hr, the toxin titre started to increase without a considerable lag period when glucose was added. The pH value decreased immediately, but then it started to increase indicating a high rate of oxidative processes. As previously stated, the differences between the final pH values of control and treated cultures did not follow a uniform pattern. At the time of addition of glucose more than 15 mg. maltose/ml. were found in the culture. The rate of utilization of the maltose was not affected by the treatment. In general, growth and the utilization of N sources were somewhat promoted but more deviations from this pattern were observed when glucose was added than with maltose. A short time after its addition, glucose could not be detected in chromatograms of the culture fluid.

Figure 3 depicts the effect of adding acetic acid 0.97 mg./ml. to the cultures at 24 hr of incubation when the culture still contained about 15 mg. maltose/ml. The general pattern is similar to that shown in the case of the addition of glucose.

DISCUSSION

It has been shown that the rate of toxin formation can be increased when certain carbon sources are added to cultures which still contain considerable amounts of maltose and which produce toxin at a high rate. The explanation offered for this finding is that, with increasing bacterial mass, the concentration of the maltose becomes suboptimal for toxin production. The fact that the cultures are able to metabolize an additional 15 mg. maltose/ml. seems to support this view. The question of how 15 times less glucose or acetic acid can cause the same or even higher increase in titres requires further investigation. It is known (Tasman & Brandwijk, 1936) that maltose is split by *C. diphtheriae* to glucose at a much slower rate than the further metabolization of glucose. Edwards (1960) could not find any sugar other than maltose in his culture filtrates possibly because the intermediate glucose was used immediately after its formation. In our experiments too, the added glucose disappeared from the cultures almost immediately after its addition. The lag in response to the addition of maltose as compared to the immediate rise in toxin titre following the addition of glucose or acetic acid, might be connected with these findings.

The fact that maltose and certain metabolism intermediates, when added at the beginning of the incubation, caused a delay in toxin formation, indicates that the ratio of concentration of nutrients to bacterial mass is of importance for high toxin formation and that nutritional requirements may change according to the physiological age of the culture. Perhaps our system was more sensitive to slight changes in pH value at the beginning of incubation rather than later. Our findings, according to which glucose, when added to the medium at 0 hr, retards toxin formation is in accordance with those of Linggood *et al.* (1947). In their system, however, much more than 1 mg. glucose/ml. was needed to obtain a pronounced adverse effect on toxin formation. This can be explained by the fact that they used much smaller and older inocula than we did, and therefore even at a high rate of glucose consumption per micro-organism the acid formation at low glucose concentrations could not reach values which would significantly influence the pH value of the cultures.

From a practical point of view, the gain in toxin yield that can be achieved by the method described, seems to be remarkable if one takes into account the low cost involved. About a hundred experiments have so far been made with the different carbon sources without the method having once failed. Fermentors offer easy technical solution for the problem of adding nutrients to growing cultures; preliminary experiments carried out on 4-litre fermentors indicate that the application of the method to such systems is feasible.

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