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Production of Staphylococcal Alpha Toxin

II. Glucose Repression of Toxin Formation

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The effect of glucose on alpha toxin production was studied in the Wood 46 strain of *Staphylococcus aureus*. Optimal toxin production occurred when 0.2% glucose was present in the medium. Omission of glucose gave lower yields of toxin, and concentrations of 0.5% and higher severely depressed toxin formation. Glucose affected the initiation of alpha toxin synthesis in growing cultures. As the glucose concentration increased, the time lag prior to the onset of toxin production also increased, and maximal rates of synthesis were not obtained until essentially all the glucose had been exhausted from the medium. The addition of glucose to toxin-producing cultures caused a temporary, almost complete repression of toxin formation which was not due to *p*H changes in the culture. The synthesis of most extracellular proteins was not inhibited during the period of repression. After recovery, toxin was produced at rates equal to those of untreated control cultures. The kinetics of toxin repression and the observation that the glucose analogues, 2-deoxy-D-glucose and α-methyl-glucoside, as well as other carbon sources, inhibit toxin production suggest that transient repression is responsible for the inhibition of toxin formation. No evidence for a regulatory role of adenosine 3′,5′-cyclc monophosphate in alpha toxin production was obtained.

The variable effect of glucose on staphylococcal alpha toxin production has been known for many years. Nélis (7) found that toxin production was enhanced by the addition of 0.1% glucose, but other investigators (4) found glucose to have an inhibitory effect. Schwartz (10) observed an initial rapid decrease in the *p*H of glucose-containing cultures, but concluded that glucose addition was unrelated to toxin production. Gladstone (5) studied alpha toxin production in a chemically defined medium and found that a concentration of 0.225% glucose gave optimal toxin production, but when glucose was omitted the growth of the cultures was impaired and the toxin concentration decreased. With glucose concentrations greater than 0.59%, no toxin was produced, although the *p*H of the culture was controlled. It appeared that the inhibition of toxin was not simply due to a decrease in *p*H, and Gladstone concluded that the inhibition was due to the accumulation of glycolytic products. More recently, de Repentigny and Mathieu (2) investigated the effects of a number of carbohydrates on alpha toxin production. They found that the glucose analogue 2-deoxy-D-glucose inhibited growth and alpha toxin production in the Wood 46 strain of *Staphylococcus aureus;* the effect on toxin was not selective, but was proportional to growth.

Morse et al. (6) studied the effects of glucose on staphylococcal enterotoxin B production. They found that toxin synthesis was initiated in the presence of glucose at *p*H values greater than 5.6, but that the differential rate of toxin production was greater when glucose was omitted from the medium. Furthermore, the addition of glucose to an actively growing culture caused an immediate inhibition in the rate of toxin production with no change in the *p*H. They concluded that enterotoxin B synthesis was regulated by catabolite repression.

We observed an inhibitory effect of glucose while studying the kinetics of alpha toxin production in strain Wood 46. Our results suggest that transient repression may play a role in the regulation of alpha toxin synthesis.

MATERIALS AND METHODS

Bacterial strains and cultural conditions. The Wood 46 strain of *S. aureus* (ATCC 10832) was used in all experiments. Stock cultures were maintained on brain heart infusion agar slants at 4 C.

The culture medium, tryptose phosphate broth (TPB), consisted of 2% tryptose (Difco), 0.5% NaCl, and unless otherwise indicated, 0.2% glucose and 0.25% Na_2HPO_4. Flasks containing 30 ml of medium were inoculated to an optical density (OD) at 540 nm of 0.05 with cells from an exponentially growing

culture and incubated at 37 C in a reciprocating shaker water bath.

Toxin assay. Alpha toxin was assayed by measuring the hemolysis of rabbit erythrocytes as previously described (3).

Extracellular protein production. Two flasks containing 30 ml of TPB were inoculated with Wood 46 cells. After 5 hr of incubation, the cultures were pooled, and 0.5 μCi of a uniformly labeled ^{14}C-amino acid mixture (New England Nuclear Corp.) per ml of culture was added. Samples of 2 ml were taken periodically and centrifuged to remove the cells, and 1 ml of supernatant fluid was added to 1 ml of cold 10% trichloroacetic acid. The precipitates were collected on membrane filters (Millipore Corp.) and placed in a scintillation fluid composed of Liquifluor (New England Nuclear Corp.) in toluene. The radioactivity present in the acid-insoluble material was measured in a Packard Tri-Carb scintillation counter.

Other determinations. The OD of cell cultures was measured at 540 nm in a Bausch and Lomb spectrophotometer. Glucose was measured enzymatically by the Glucostat method (Worthington Biochemical Corp.).

RESULTS

Effect of glucose concentration on cell growth and alpha toxin production. The variable effect of glucose on toxin production was observed when Wood 46 cells were inoculated to an OD of 0.05 into TPB containing various concentrations of glucose. The cultures were incubated for 12 hr and assayed for toxin titer, *p*H, and cell growth. A typical experiment is shown in Table 1. We consistently observed that highest toxin yields were produced at a concentration of 0.2%, although cell growth was greatest at 0.35%. Less toxin was produced when glucose was omitted from the medium, and glucose concentrations of 0.5% and higher severely depressed toxin production.

Effect of glucose on initiation of toxin production. A previous investigation of the kinetics of alpha toxin formation revealed that toxin was produced by Wood 46 cultures only after a "lag" period of several hours (3). During this period in which the cells grow exponentially, no extracellular toxin can be detected. To test whether the cells from a culture in the latter part of the lag period would synthesize toxin without delay in fresh medium, we removed cells from a 5-hr culture by centrifugation and suspended one sample in fresh TPB and another in an equal volume of the same 5-hr supernatant fraction. Samples were removed periodically for OD and toxin determinations. The results (Fig. 1) showed that the control cells suspended in 5-hr supernatant fractions began to make toxin without delay, but cells suspended in fresh medium produced toxin at a much lower rate. Since we had found that glucose had a variable effect on toxin production in our 12-hr cultures (Table 1), we suspended a third sample of cells in fresh TPB from which glucose had been omitted. These cells produced toxin at the same rate as the control cells.

The inhibitory effect of glucose on the initiation of toxin synthesis was further examined by inoculating cells to an OD of about 0.05 in TPB containing glucose at concentrations of 0, 1.5, 3.0, or 5.5 mg/ml. Samples were removed periodically and assayed for toxin and glucose concentration. The results in Fig. 2A show that with increasing concentrations of glucose there was an increasing delay before maximal rates of toxin synthesis were reached. Toxin production began very early (120 min) in the culture containing no glucose, but the rate of synthesis diminished after only 2 hr. An examination of the glucose content

TABLE 1. *Effect of glucose concentration on cell growth and alpha toxin production in Staphylococcus aureus*

Glucose concn (%)	OD_{540}	*p*H	Toxin (hemolytic units/OD_{540})
None	7.54	8.05	100
0.10	8.64	7.75	232
0.20	9.90	7.45	242
0.35	10.56	6.90	149
0.50	8.74	5.41	33
0.70	8.74	5.12	13

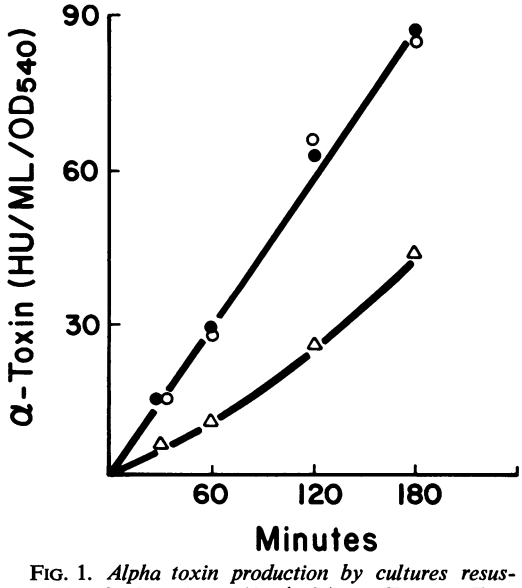

FIG. 1. *Alpha toxin production by cultures resuspended in fresh medium. TPB (5 hr), ●; TPB △; TPB without glucose, ○.*

of the cultures (Fig. 2B) revealed that maximal rates of toxin production were reached only after virtually all of the glucose had been exhausted from the medium.

Effect of glucose added to cultures actively producing toxin. To determine the effect of glucose once toxin synthesis had begun, flasks of TPB were inoculated to an OD of 0.06, and after incubation for 4 hr the flasks were pooled, and the culture was divided into three portions. The first served as the control, the second received glucose to a concentration of 0.3%, and the third received the same amount of glucose and, in addition, received drops of 0.1 N NaOH periodically to maintain the same *p*H as that of the control culture. Samples were removed at various times, and OD, *p*H, and toxin titer were determined. The addition of glucose alone caused a complete inhibition of toxin production (Fig. 3A). When the *p*H of the glucose culture was maintained at the control culture level, an early inhibition of toxin production was still observed. An examination of the growth of these cultures (Fig. 3B) showed that for approximately 120 min the glucose-treated culture kept at the control *p*H increased in OD more rapidly than did the control. Since this rapid growth of the glucose-treated culture would tend to minimize any inhibitory effect on toxin as shown in Fig. 3A, the differential rate of toxin synthesis in glucose and control cultures was determined by plotting toxin production as a function of growth. The results (Fig. 4) showed that toxin production in the glucose-*p*H-maintained culture was completely repressed and that the culture grew very rapidly during the period of inhibition. Toxin production resumed once glucose had been exhausted (Fig. 3A) and continued at a rate slightly greater than in the control culture.

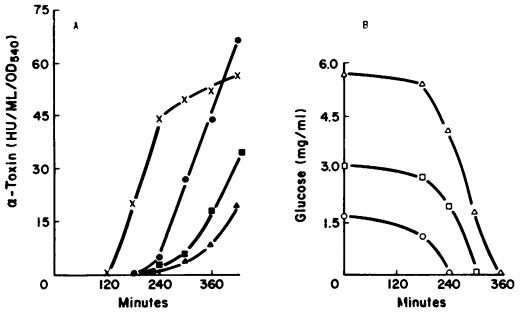

FIG. 2. *Effect of glucose on the initiation of toxin synthesis. No glucose,* ×; *0.15% glucose,* ●, ○; *0.3% glucose,* ■, □; *0.55% glucose* ▲, △.

Effect of glucose on the synthesis of extracellular proteins. To determine whether glucose inhibited the production of all extracellular proteins during the period of maximal toxin repression, 5-hr cultures were labeled with a ^{14}C-amino acid mixture as described above. Half of the culture received glucose and Na_2HPO_4 to concentrations of 0.25%, and the remaining half served as the control. Samples were removed periodically, and the OD, toxin titer, and amount of acid-insoluble radioactivity in the extracellular fluid were determined. There was some difference in the amount of labeled extracellular protein in control and glucose-treated cultures (Fig. 5), but it is apparent that glucose does not inhibit the production of all extracellular proteins. The inset in Fig. 5 shows toxin repression by glucose under these experimental conditions.

Effect of other carbohydrates on alpha toxin

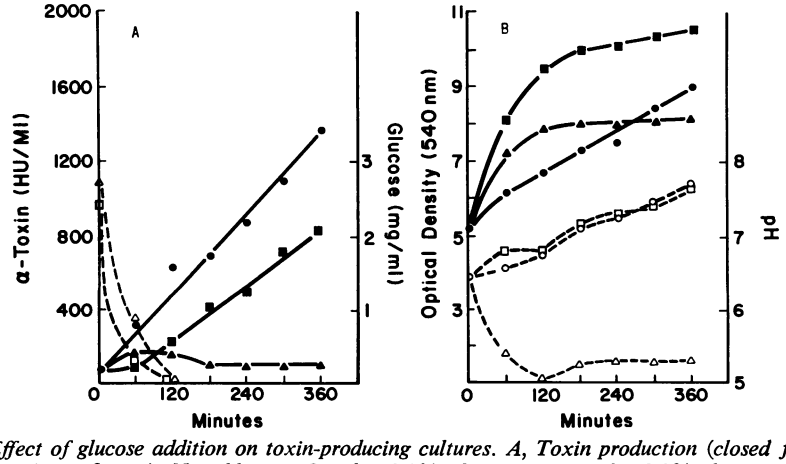

FIG. 3. *Effect of glucose addition on toxin-producing cultures. A, Toxin production (closed figures) and glucose utilization (open figures). No additions,* ●; *plus 0.3% glucose* ▲, △; *plus 0.3% glucose and 0.1 N NaOH,* ■, □. *B, Cell growth (closed figures) and pH (open figures). Symbols as in A.*

production. Wood 46 cells which had been growing in TPB for 4.5 hr were pooled, and various carbohydrates were added to a concentration of 0.3% to samples of the culture. A sample was removed for OD and toxin determinations, and the cultures were incubated for 90 min. During this period, the *p*H of each culture was maintained at the control culture *p*H by adding drops of NaOH. A second sample was taken at the end of the incubation period, and the changes in toxin titer and OD were determined. Glucose caused an 85 to 95% inhibition of toxin production during this period (Table 2); other carbohydrates gave varying amounts of inhibition, the greatest being just over 50%. Two non-metabolizable analogues of glucose, α-methyl-glucoside, and 2-deoxy-D-glucose also inhibited alpha toxin production.

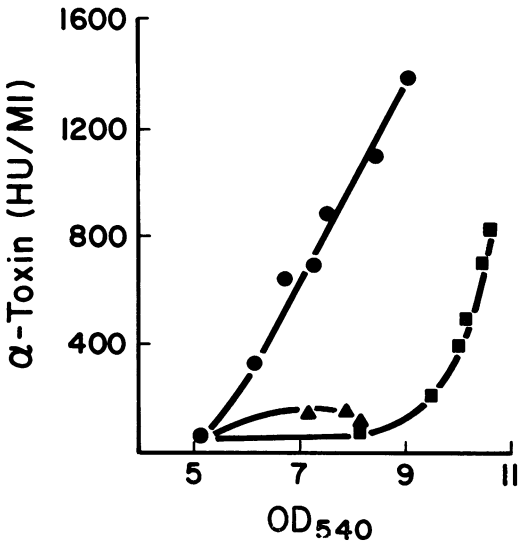

FIG. 4. *Glucose repression of alpha toxin production. Symbols: as in Fig. 3A.*

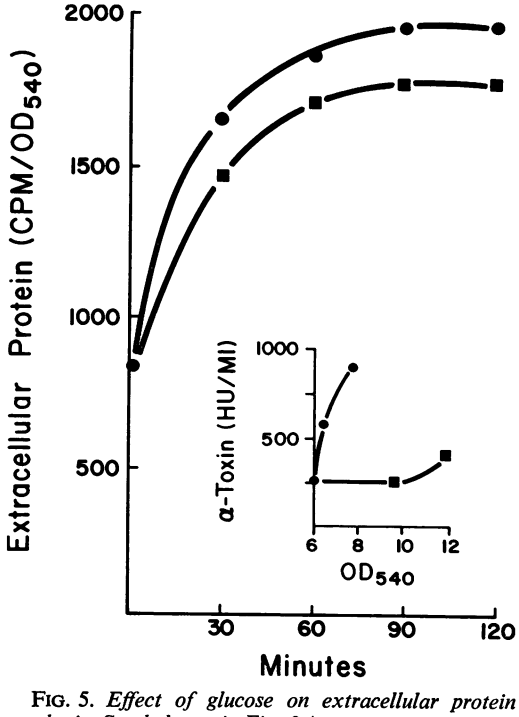

FIG. 5. *Effect of glucose on extracellular protein synthesis. Symbols: as in Fig. 3A.*

Role of C-AMP in the glucose effect. The results thus far suggested that the glucose effect on toxin production was similar to catabolite or transient repression, or both. Since cyclic adenosine monophosphate (C-AMP) is known to be involved in both types of repression (8, 9), we examined the possibility that the effects we observed on alpha toxin synthesis were mediated by this compound. However, all attempts to overcome toxin repression by adding 0.05 M C-AMP to glucose-treated cells were unsuccessful.

Effect of pH and temperature on alpha toxin production. Glucose causes a repression of alpha toxin production which is not due to a *p*H change in the culture. However, since the addition of glucose alone caused the *p*H to drop to around 5.3 (Fig. 3), we wanted to determine whether toxin production was compatible with this H^+ concentration. A 4-hr culture was divided into three fractions. The first received NaH_2PO_4 to 0.25% and HCl to bring the *p*H to 5.5, the second received Na_2HPO_4 to 0.25% and NaOH to raise the *p*H to 8.0, and the third sample served as the

TABLE 2. *Effect of carbon sources on alpha toxin formation*

Addition[a]	Δ Toxin[b]/Δ Mass[c]	Inhibition (%)
None	233.9	
Glucose	34.4	85
Maltose	110.0	53
Glycerol	115.0	51
None	191.1	
Glucose	30.4	84
Galactose	154.9	19
Lactose	168.2	12
None	192.1	
Glucose	5.1	97
α-Methyl-glucoside	152.0	21
2-Deoxy-glucose	88.8	54

[a] Carbon sources were added to TPB to a concentration of 0.3%.
[b] Hemolytic units.
[c] OD_{540}.

control. The three cultures were incubated for 5 hr during which time the first two cultures were maintained at *p*H 5.5 and 8.0, respectively. Toxin and OD determinations during this time indicated that very little toxin was made at these *p*H values (Fig. 6).

We also examined toxin production in TPB cultures incubated at 37, 30, and 20 C. Toxin was not produced at 20 C and was produced very slowly at 30 C, almost reaching the 37 C level after 22 hr of incubation.

DISCUSSION

The results of this investigation confirm earlier reports of the variable effect of glucose on staphylococcal alpha toxin production. Optimal toxin synthesis occurred in TPB cultures containing 0.2% glucose. Toxin production was initiated earlier when glucose was omitted from the medium, but overall cell growth and toxin concentration were lower than in 0.2% glucose cultures. Thus, glucose at low concentrations stimulates toxin synthesis, presumably by acting as an energy source for the cells. On the other hand, glucose at concentrations of 0.5% and higher depressed toxin synthesis dramatically while having only a slight effect on cell growth. The effect at high concentrations of glucose is probably due to the rapid decrease in *p*H to levels which are incompatible with alpha toxin production; little toxin is produced at *p*H 5.5. This variable effect of glucose is not unique to toxin production. A dualistic effect of glucose has been reported in studies of mannosidase production as well as in other inducible enzyme systems (1).

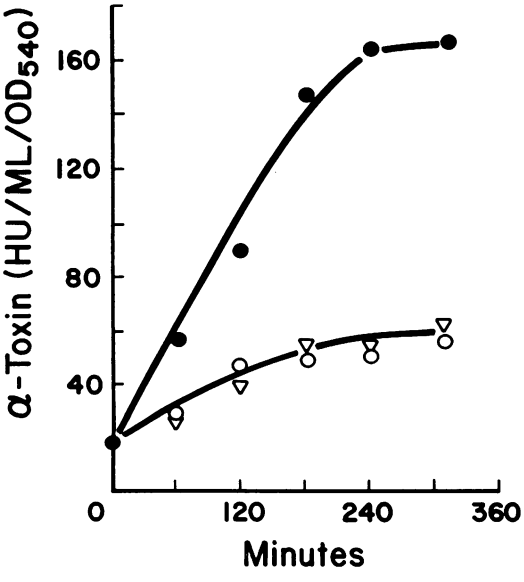

FIG. 6. *Effect of pH on alpha toxin production. Control culture, ●; pH 5.5 culture, ▽; pH 8.0 culture, ○.*

Glucose also causes a temporary, severe inhibition of toxin production, which seems to be unrelated to any effect on the *p*H of the culture. This inhibitory effect is seen in cultures maintained at the control culture *p*H and appears to last until glucose has been exhausted from the medium. When toxin synthesis resumes, the rate of production is equal to, or slightly greater than, nontreated control rates. Several features of the glucose effect seen here suggest that a process related to the phenomenon known as transient repression may be responsible for the inhibition of toxin formation. The kinetics of toxin repression are very similar to those observed in transient repression by Tyler et al. (11) in their study of β-galactosidase synthesis in an *Escherichia coli* mutant (LA-12G) which is insensitive to catabolite repression. They found that the addition of glucose (or glucose and glycerol) almost completely repressed enzyme synthesis in cultures of this mutant, and, when the cells recovered, β-galactosidase was produced at the same differential rate as in their untreated control cultures. Another finding which suggests transient rather than catabolite repression is the inhibition of alpha toxin production by glucose analogues as well as by other carbon sources (11, 12). The analogues are apparently transported and phosphorylated, and this is sufficient to repress toxin synthesis without further catabolism. We also observed (not shown) that 2-deoxy-glucose and α-methyl-glucoside had some inhibitory effect on cell growth (2). Although C-AMP is known to mediate transient repression in gram-negative cells, we were unable to demonstrate any regulatory role of this cyclic nucleotide in toxin production. The failure to relieve toxin repression by the addition of C-AMP to glucose-treated cultures could be due to the inability of the molecule to enter the cells.

Our results also indicate that the glucose effect exhibits some specificity, in that the production of all extracellular proteins is not inhibited during the transient repression of toxin. We do not know whether the difference in extracellular proteins present in control and glucose-treated cultures shown in Fig. 5 can be entirely accounted for by the inhibition of alpha toxin, or whether some other staphyloccal products are also repressed. At any rate, it would appear that most extracellular proteins continue to be synthesized during the transient repression of alpha toxin.

ACKNOWLEDGMENTS

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