Regulation of Staphylococcal Enterotoxin B: Effect of Thiamine Starvation

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During the transition between the exponential and stationary phases of growth, there was a rapid accumulation of both cell-associated and extracellular enterotoxin B. Extracellular enterotoxin was synthesized until the cells entered the stationary phase during which cell-bound toxin was not detected. The differential rate of toxin synthesis relative to that of total protein synthesis was greater at pH 7.7 than at 6.0. Addition of glucose decreased the differential rate of toxin synthesis. This decrease was greater at pH 7.7 than at 6.0. Addition of pyruvate decreased the differential rate at pH 7.7 but not at 6.0. Analysis of the non-gaseous end products of glucose and pyruvate metabolism showed that conditions which favor the oxidative decarboxylation of pyruvate also favor the repression of toxin synthesis. Elimination of thiamine from the medium prevented the oxidative decarboxylation of pyruvate by Staphylococcus aureus S-6 and partially or completely reversed the repression of toxin synthesis by glucose and pyruvate. In the absence of an added energy source, thiamine starvation caused a decrease in protein synthesis but an increased differential rate of toxin synthesis which was greater at pH 7.7 than at 6.0. In the absence of thiamine, pyruvate was not metabolized but caused a decrease in the rate of protein synthesis. This resulted in a twofold increase in the differential rate of toxin synthesis. Thus, conditions which altered the oxidative decarboxylation of pyruvate or decreased the rate of protein synthesis increased the rate of enterotoxin B synthesis.

Recent investigations on the kinetics of enterotoxin formation revealed that toxin was not detected in the culture medium until exponential growth had terminated (13, 16, 18). Maximum synthesis occurred during the transition from exponential to stationary phase growth (18). Our preliminary studies (S. A. Morse, R. A. Mah, and W. J. Dobrogosz, Bacteriol. Proc., p. 3, 1968; S. A. Morse and R. A. Mah, Bacteriol. Proc., p. 10, 1969) demonstrated that the synthesis of enterotoxin B by Staphylococcus aureus S-6 was severely repressed when glucose was added to the medium. Metabolism of glucose resulted in a marked decrease in pH. Recently, we have shown (18) that the repression of toxin synthesis was not due to the decrease in pH resulting from the incomplete oxidation of glucose. The present study is concerned with whether this repression is altered by conditions which change the metabolism of pyruvate formed during the catabolism of glucose.

MATERIALS AND METHODS

Organism. S. aureus S-6 was obtained from M. S. Bergdoll (Food Research Institute, University of Wisconsin). This strain produces large amounts of enterotoxin B and small amounts of enterotoxin A. Storage of the culture, preparation of inocula, and incubation conditions have been described previously (14, 18).

Medium. The basal medium consisted of a 1% solution of casein hydrolysate (PHP, Mead Johnson International, Evansville, Ind.) supplemented with thiamine (33 ng/ml) and nicotinic acid (1.2 μg/ml). This solution is referred to as the PHP medium. For studies on the effect of thiamine, the basal medium consisted of a 1% solution of vitamin-free Casitone (Difco) supplemented with nicotinic acid (1.2 μg/ml). This solution is referred to as the nicotinic acid-Casitone (NAC) medium.

Thiamine-deficient cells. A culture grown aerobically in 2% PHP for 12 hr was chilled and washed twice by centrifugation in cold, sterile distilled water. The cells were resuspended to their original volume, and 5 ml was used to inoculate 500 ml of NAC medium. This culture was incubated for 5 hr at 37°C on a gyratory shaker, and the cells were harvested by centrifugation (20,000 × g for 15 min). Cells were concentrated (0.8 to 0.9 mg/ml) by resuspending to one-sixth their original volume in 0.1 M phosphate-buffered NAC medium, pH 7.

Determination of end products. Nongaseous end products of glucose and pyruvate metabolism were determined by column chromatography on silicic acid with various concentrations of t-butanol in.
choloroform as solvents. The technique used for the preparation and packing of the silicic acid and preparation of solvents was essentially that of Ramsey (19). Preparation of sample and elution procedures were those reported by Dobrogosz (4). Glucose-1-14C (0.24 μCi/ml) or pyruvate-1-14C (0.24 μCi/ml) was added at zero time. Nonlabeled glucose or pyruvate was added at a concentration of 0.5%. Cultures were incubated for 2 hr before samples were collected.

Known samples of 14C-labeled ethanol, acetate, formate, pyruvate, lactate, and succinate were chromatographed to determine their elution position. Acetoin was determined chemically by the method of Westerfeld (27). In all cases, a consistent elution profile was obtained.

**Determination of extracellular enterotoxin.** Samples for toxin assay were prepared as previously described (18). Enterotoxin was determined by the single-gel diffusion method of Weirother et al. (26). Reference standards containing 12.5, 25, 50, 100, and 200 μg of toxin/ml were prepared by diluting purified enterotoxin B in phosphate-buffered saline, pH 7.4. A reference curve was plotted for each experiment. The gel-diffusion tubes were incubated in a water bath at 30°C and read at 24 and 48 hr.

**Determination of cell-bound toxin.** Cell-bound toxin was estimated by using specific antiserum conjugated with fluorescein isothiocyanate at a dye-protein ratio of 1:40 (22). The unreacted dye was removed by overnight dialysis at 4°C against 0.0175 m phosphate buffer, pH 6.3. The conjugated antiserum was then fractionated on a diethylamincelullose column prepared by the method of Levy and Sober (12) and eluted with a gradient of sodium chloride (0.01 to 0.05 m) in 0.0175 m phosphate buffer, pH 6.3 (21). The conjugated antiserum was tested with strains of *S. aureus* capable of synthesizing only enterotoxin A and C as well as with nonenterotoxigenic isolates from strain S-6. The conjugated antiserum reacted specifically with only the enterotoxin B-producing strain, S-6.

Samples were removed at hourly intervals from an aerated culture grown under previously described conditions (18) and centrifuged (20,000 × g for 15 min) to remove the cells. The packed cells were used to make a smear which was air-dried and fixed in 95% ethyl alcohol for 1 min. Fixed smears were flooded with conjugated antiserum and incubated for 30 min in a moist chamber at 37°C. The slides were rinsed in 0.0175 m phosphate buffer for 10 min and then examined with a Leitz microscope, using an Osram HBO 200 W maximal-pressure mercury-vapor arc with UG 1 exciter filter and BG 12 and BG 38 barrier filters. Cells were arbitrarily given values of +4, +3, +2, +1, and 0 based on the degree of cell fluorescence. Strain S-6 grown under aerobic conditions for 6 hr in 1% PHP medium was used as the +4 control.

**Radioisotopes.** Uniformly labeled leucine (leucine-1-14C), uniformly labeled glucose (glucose-6-14C), sodium acetate-1-14C, 1-14C-sodium formate, di-sodium lactate-1-14C, sodium pyruvate-1-14C, and succinic acid-1,4-14C were obtained from New England Nuclear Corp. (Boston, Mass.). Ethyl alcohol-1-14C was a product of International Chemical and Nuclear Corp. (City of Industry, Calif.). Pyruvate-1-14C was obtained from Amersham/Searle Corp. (Arlington Heights, Ill.).

**Chemicals.** 2,5-Diphenyloxazolozole (POPO) and 1,4-bis-2-(5-phenyloxazoloyl) benzene (POPOP) were obtained from Packard Instrument Co. (Downers Grove, Ill.). All other reagents were analytical grade.

**Miscellaneous measurements.** Glucose was determined enzymatically by the glucostat method (Worthington Biochemical Corp., Freehold, N.J.). Total protein synthesis was measured as previously described (18). Radioactivity of samples was measured in a liquid scintillation spectrometer (Packard Instrument Co.) after addition of 15 ml of scintillation fluid (0.4% POPO and 0.01% POPOP in toluene). Turbidity was measured by Klett-Summerson colorimetry at 540 nm.

**RESULTS**

**Kinetics of toxin synthesis.** The relationship between cell-associated and extracellular enterotoxin B was determined during the growth of strain S-6 in unbuffered 1% PHP medium. The results (Fig. 1) show that toxin was not detected during exponential growth. As the culture entered the transition between the exponential and stationary phases of growth, there was a rapid accumulation of cell-associated enterotoxin coupled with its appearance in the culture medium. Extracellular enterotoxin accumulated rapidly in the medium until the cells ceased to fluoresce. Toxin was no longer synthesized after the culture entered the stationary phase of growth.

**Effect of pyruvate and glucose.** Previous results (18) showed that the differential rate of enterotoxin synthesis between aerobic and anaerobic cultures was due to the availability of oxygen at the expense of the carbon source used. The experiments described in this study were performed under aerobic conditions and in the presence of glucose. The importance of the carbon source was evaluated using pyruvate and glucose.

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**Fig. 1. Relationship between cell-associated and extracellular enterotoxin B.** Symbols: O, turbidity; △, extracellular enterotoxin B (μg/ml); solid line, cell-associated enterotoxin B (percent relative fluorescence).
Table 1. Effect of glucose and pyruvate on the differential rate of enterotoxin B synthesis by Staphylococcus aureus S-6

| Additions to PHP medium | Activity at pH 6.0 | Activity at pH 7.7 |
|-------------------------|-------------------|-------------------|
|                         | Differential rate | Per cent repressed | Differential rate | Per cent repressed |
| None.................... | 1.81              |                    | 3.10              |                    |
| Glucose................ | 1.24              | 0.34              | 1.31              | 68                |
| Pyruvate..............  | 1.92              | 1.52              | 51                |                    |

* Casein hydrolysate supplemented with thiamine and nicotinic acid.

Values to be multiplied by 10^-3.

Toxin B synthesis by PHP-grown cells of S. aureus S-6 was exponential. However, linear differential rates were obtained when the initial period of toxin synthesis was examined. The differential rates observed when cells were resuspended in buffered PHP medium are shown in Table 1. In the absence of any added energy source, the rate of synthesis was greater at pH 7.7 than at 6.0. When glucose was present, the differential rate was repressed. This repression was twice as great at pH 7.7 than was observed at 6.0. Glucose uptake was similarly affected. Rates were greater at pH 7.7 than at 6.0 (0.176 and 0.147 umole per ml per min, respectively) but this difference (16%) would not account for the twofold increase in repression.

The difference in repression may have resulted from changes in the metabolism of glucose which could be demonstrated by alterations in the proportions or types of end products formed. Examination of the nongaseous end products of glucose metabolism (Fig. 2) revealed that the pH of the medium affected the dissimilation of the pyruvate formed during its catabolism. At pH 6.0, the pyruvate formed from glucose was dissimilated to acetate and CO2 with the NADH2 reoxidized via the formation of acetoin. Lactate was a minor product. At pH 7.7, pyruvate accumulated in the medium but also was dissimilated to acetate and CO2. However, the amount of lactate and other reduced products was not sufficient to account for the complete reoxidation of the NADH2. Therefore, it appears that the majority of the NADH2 was reoxidized via some other mechanism, probably the electron transport system. The tricarboxylic acid cycle did not participate in the catabolism of glucose as shown by radiorespirometric data (S. A. Morse, Ph.D. Thesis, Univ. of North Carolina, Chapel Hill, 1969). Carbon dioxide was postulated as the initial C1 compound as formate was not present in detectable amounts. However, the data do not rule out the possibility that some formate is formed and then subsequently oxidized (11).

The addition of pyruvate to PHP medium buffered at pH 6.0 had no effect on the differential rate of toxin synthesis (Table 1). However, at pH 7.7 pyruvate repressed the rate of synthesis by 51%. This repression was somewhat less than that observed with glucose. Figure 3 shows that, at pH 6.0, pyruvate was dissimilated to acetate (and CO2) coupled with the formation of acetoin to regenerate nicotinamide adenine dinucleotide+ (NAD+). At pH 7.7, pyruvate was also dissimilated to acetate and CO2, but the presence of formate may indicate that a portion of the CO2 arose from the metabolism of this compound. Under these conditions, the amount of end products, such as lactate, was not sufficient to account for the reoxidation of the NADH2 formed during the oxidative decarboxylation of pyruvate or the oxidation of formate. Succinate (data not shown) appeared as a minor end product of pyruvate metabolism. The accumulation of acetate indicated that tricarboxylic acid cycle activity was low and that, in the presence of amino acids, this pathway was primarily used for biosynthetic purposes.

**Effect of thiamine on glucose and pyruvate metabolism.** Nongaseous end products resulting from the metabolism of glucose by thiamine
deficient cells of *S. aureus* S-6 are shown in Fig. 4. At pH 6.0, glucose was utilized slowly in the absence of thiamine and was dissimilated almost entirely to pyruvate. When thiamine was added, acetoin and acetate increased, whereas pyruvate decreased, thus confirming that this cofactor was required for their formation in strain S-6. Without thiamine, glucose was more rapidly utilized at pH 7.7 than at 6.0 and was also converted almost entirely to pyruvate. When thiamine was present, the pattern of end products shifted dramatically toward acetate formation. The concomitant increase in lactate indicated that some of the NADH$_2$ was reoxidized via lactate dehydrogenase.

Pyruvate metabolism by thiamine-deficient cells was similarly affected (Fig. 5). Without thiamine, pyruvate could not be used as an energy source at either pH 6.0 or 7.7. In the presence of thiamine at pH 6.0, pyruvate was dissimilated to acetate, lactate, and a small amount of acetoin. At pH 7.7, acetate and lactate were the predominant products.

**Effect of thiamine on protein and enterotoxin B synthesis.** In the absence of an added energy source, thiamine starvation produced a marked decrease in protein synthesis and was accompanied by an increased differential rate of toxin synthesis (Table 2). This increase was greater at pH 7.7 than at 6.0. The amount of toxin synthesized was doubled even though protein synthesis decreased by 50% from pH 6.0 to 7.7. When thiamine was present, the rate of protein synthesis increased, but, because the synthesis of toxin did not increase proportionately, the differential rate decreased.

Without thiamine, the cells were able to catabolize glucose as far as pyruvate. The limited amount of energy obtained by this dissimilation caused an increase in the rate of protein synthesis. A marked difference in the differential rates of toxin synthesis was observed between pH 6.0 and 7.7. When compared with the control value, the rate of synthesis was more repressed at pH 7.7 than at 6.0. When thiamine was added in the presence of glucose, protein synthesis increased. Under these conditions, the repression of toxin synthesis at pH 7.7 was approximately 50% greater than in the absence of thiamine. The addition of thiamine did not significantly alter the differential rate at pH 6.0.

Pyruvate could not be catabolized in the absence of thiamine. Its addition resulted in a more severe inhibition of protein synthesis than was observed in the absence of any added energy source and a twofold increase in the differential rate of toxin synthesis. The reason for this effect is not apparent and is presently under investigation. Although the addition of thiamine stimulated the
rate of protein synthesis at both pH values, the differential rate of toxin synthesis was only repressed at pH 7.7.

An inverse relationship was observed between total protein synthesis and the differential rate of enterotoxin B synthesis (Fig. 6). Conditions which decreased the rate of protein synthesis increased the differential rate of toxin synthesis. Conversely, conditions which increased the rate of protein synthesis decreased the differential rate of toxin synthesis. This effect was more evident at pH 7.7 than at 6.0.

**DISCUSSION**

Unlike the synthesis of enterotoxin A which occurred during exponential growth (17), the formation of enterotoxin B was not detected until this growth phase terminated. No intracellular enterotoxin has been detected during exponential growth (5, 15, 16). These characteristics of enterotoxin B synthesis are similar to those reported for the biosynthesis of secondary metabolites (25) and several other bacterial exotoxins (20). We can advance several hypotheses to explain this lag in toxin formation. The first possibility is that during exponential growth a metabolite accumulated in the medium or cells and induced the synthesis of enterotoxin B. This induction hypothesis can be eliminated on the basis of data reported by Markus and Silverman (16) which showed that propagation of cells in a medium taken from cultures just prior to maximum toxin production could not eliminate the lag. In addition, inoculation of washed stationary phase cells into this partially exhausted medium, adjusted to pH 7.0, resulted in the initiation of a new growth cycle with normal kinetics of toxin synthesis.

Another possibility was suggested by Coleman (3) who proposed that a limitation of exoenzyme

**TABLE 2. Effect of glucose and pyruvate on the differential rate of enterotoxin B synthesis by thiamine-starved cells of Staphylococcus aureus S-6**

| Additions to NAC medium* | Leucine-\(^{14}C\) (counts per min per ml) | Enterotoxin B µg/ml | Differential rate\(^b\) | Per cent of thiamine control |
|-------------------------|------------------------------------------|----------------------|--------------------------|------------------------------|
|                         | pH 6.0 | pH 7.7 | pH 6.0 | pH 7.7 | pH 6.0 | pH 7.7 | pH 6.0 | pH 7.7 | pH 6.0 | pH 7.7 |
| None                    | 1,443  | 758    | 2.46   | 4.99   | 1.70   | 6.58   | 113    | 164    |
| + thiamine              | 2,749  | 1,788  | 4.11   | 7.13   | 1.50   | 3.99   | 100    | 100    |
| Glucose                 | 1,956  | 1,940  | 2.22   | 3.83   | 1.13   | 1.97   | 75     | 49     |
| + thiamine              | 3,354  | 3,675  | 3.62   | 4.05   | 1.08   | 1.10   | 72     | 27     |
| Pyruvate                | 833    | 463    | 2.44   | 3.84   | 2.93   | 8.30   | 195    | 208    |
| + thiamine              | 3,472  | 2,980  | 5.51   | 8.21   | 1.59   | 2.76   | 106    | 69     |

* Nicotinic acid-Casitone medium.
\(^b\) Values to be multiplied by \(10^{-3}\).
messenger ribonucleic acid (mRNA) synthesis caused by depletion of the nucleic acid precursor pool during rapid ribosomal RNA synthesis accounted for the characteristic lag in exoenzyme synthesis. After a decrease in the growth rate and ribosomal RNA synthesis, the precursor pool would increase in size and allow exoenzyme mRNA synthesis to proceed at a maximal rate. These findings may not apply to enterotoxin B formation since the enzymes measured in this investigation were also synthesized at low levels during exponential growth. However, because these enzyme assays are more sensitive than the serological assay used to measure enterotoxin B, there exists the possibility that small amounts of toxin are also formed during exponential growth. Therefore, this hypothesis cannot be completely eliminated.

An alternate explanation is that toxin synthesis is derepressed at the end of exponential growth. This may occur by a decrease in the level of a corepressor or by the breakdown of an unstable protein repressor molecule. The data obtained in this and other related investigations favors this model for the control of enterotoxin B synthesis in *S. aureus* S-6.

Gallant and Stapleton (6) reported that the derepression of alkaline phosphatase synthesis in *Escherichia coli* was due to an unstable repressor molecule. The synthesis or degradation of this repressor was temperature-dependent since repression was reestablished faster in derepressed cells at 32°C than at 37°C. Observations of temperature-dependent synthesis of enterotoxin B were reported by Marland (Ph.D. Thesis, Univ. of North Carolina, Chapel Hill, 1966) who showed the amount of toxin synthesized by cultures grown to the same cell density at 15.2 and 37.0°C was 120-fold greater at 37.0°C than at 15.2°C. Markus and Silverman (16) reported that cells of strain S-6 initially grown at 20°C and resuspended in medium at 37°C synthesized toxin at one-sixth the rate of cells initially grown at 37°C. In addition, cells initially grown at 37°C and resuspended in medium at 20°C synthesized one-tenth the amount of toxin as those resuspended in medium at 37°C. These findings may indicate that a temperature-sensitive repressor similar to that reported for alkaline phosphatase (6) is involved in the control of enterotoxin B synthesis.

Conditions which affected toxin synthesis also altered the metabolism of glucose and pyruvate. Previous investigations of glucose and pyruvate metabolism by *S. aureus* were primarily concerned with their metabolism by resting cell suspensions. Under these conditions, acetate and CO₂ were the principal end products (7). The present study shows that in growing cells the metabolism of pyruvate, whether derived from glucose or added as the energy source, was pH-dependent. A similar pH effect was previously reported in *E. coli* (1). In *S. aureus* S-6, the pH appeared to affect the mechanism for regeneration of NAD⁺. Even though an NAD-specific lactate dehydrogenase was present in cells of *S. aureus* grown under similar conditions (8), it did not actively participate in the reoxidation of NADH₂ as evidenced by the low amount of lactate formed. The formation of acetoin by the reduction of diacetyl is a mechanism through which NADH₂ may be oxidized. A similar pathway was proposed for *S. aureus* (2, 23) although acetoin formation via the decarboxylation of α-acetolactate was also reported (24). The lack of acetoin at pH 7.7 may indicate that the enzymes required for its synthesis are not active at this pH. However, it is also possible a competition for thiamine pyrophosphate or NADH₂ (9) would produce a similar effect. In the absence of acetoin formation, the electron transport system is the most likely mechanism for regeneration of NAD⁺ (8).

The absence of thiamine markedly affected the metabolism of pyruvate. The dissimilation of pyruvate was thiamine-dependent since little or no acetate was formed. The end products resulting from the metabolism of pyruvate by thiamine-starved cells in the presence of thiamine did not resemble identically the pattern observed with unstarved cells. These results could be explained if...
the addition of thiamine induced the synthesis of some thiamine-dependent enzymes as was reported in *Saccharomyces cerevisiae* (28). The observed decrease in the amount of acetate and acetoin would result because of the time required before the enzymes increased sufficiently to alter the pattern of end products.

The relationship between glucose or pyruvate metabolism and toxin synthesis is still not clear. However, conditions which favor the oxidative decarboxylation of pyruvate also favor the repression of toxin synthesis. Results of studies on the mechanism of this inhibition are currently being prepared for publication.

Horowitz et al. (10) have recently proposed a model to explain the repression of tyrosinase synthesis in rapidly growing cultures of *Neurospora crassa* and its subsequent derepression when protein synthesis was altered by fasting or antibiotics. A similar model could account for many of the observations on the enterotoxin B system. During exponential growth, toxin synthesis is controlled by an unstable, continuously synthesized, protein repressor. As the rate of protein synthesis decreases due to the transition from exponential to stationary phase growth, or to the lack of an essential growth factor such as thiamine, the rate of degradation of the repressor exceeds its rate of formation and toxin synthesis is derepressed. The activity of the repressor is affected by environmental conditions and is enhanced at low temperature or at low pH. The addition of a rapidly metabolizable energy source, such as glucose or pyruvate, increases the degree of repression. The effect is most evident at high pH. Since metabolism of the compound is necessary for repression, the effect could result from the interaction of a common metabolite with the repressor which may either alter its rate of degradation or increase its activity.

A possible alternative explanation is that the rapid metabolism of the energy source at high pH leads to an excess of energy which would increase the rate of protein synthesis and hence the rate of repressor synthesis. The experimental data do not permit the distinction at this time between repression at the transcriptional or translational levels. A clarification of the site of repression is currently under investigation.

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