INFECTION AND IMMUNITY Feb. 1974, p. 229-235

Vol. 9, No. 2
Printed in U.S.A.

Staphylococcal Enterotoxin Synthesis During the Exponential, Transitional, and Stationary Growth Phases

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Received for publication 5 March 1973

Small inocula (1 to 10 colony-forming units per ml of broth) of *Staphylococcus aureus* strains S-6, S-6R, and FRI-100 were employed to study growth and enterotoxin synthesis in 4% protein hydrolysate powder broths. For each strain, the exponential growth phase ended once the population approached 10^9 to 2×10^9 colony-forming units per ml. By that time, the concentrations of enterotoxins A and B reached the minimal level (1 to 2 μg/ml) at which the single gel diffusion tube method becomes applicable. By microslides and reverse passive hemagglutination, enterotoxins A and B were found to be synthesized during the exponential growth phase, but at different exponential rates.

The relationship of enterotoxin synthesis to growth is unclear. Several investigators have shown that high concentrations of enterotoxin are detected only in broths where growth has been abundant (5, 6, 10, 15, 19). Where growth is adversely affected by additives or growth conditions, enterotoxin synthesis is also adversely affected; however, good growth is not necessarily accompanied by good toxin synthesis.

Wu and Bergdoll (21) showed that enterotoxin B synthesis was stimulated by various fractions of protein hydrolysate powder (PHP). All of these fractions, however, also stimulated growth. In a later paper concerning the relationship of enterotoxin B synthesis to growth, they (22) presented a synthetic medium in which the synthesis of enterotoxin B paralleled growth until several amino acids had been depleted. Thereafter, growth continued for several hours, whereas toxin synthesis did not.

Markus and Silverman (11, 12) attempted to show that enterotoxins A and B were, respectively, what they called primary and secondary metabolites. Morse et al. (14), monitoring enterotoxin B synthesis during diauxic growth, detected toxin after the glucose in their PHP broth had been completely oxidized. They suggested that enterotoxin B was regulated by catabolite repression. In a subsequent paper concerning the utilization of pyruvate, Morse and Baldwin (13) advanced the hypothesis that enterotoxin B synthesis is derepressed at the end of the exponential growth phase. The possibility that nondetectable quantities were present prior to that time was not completely discounted.

In this paper, we present data obtained by single gel diffusion, microslides and reverse passive hemagglutination that show that enterotoxins A and B are both produced during all phases of growth.

MATERIALS AND METHODS

Organisms. The following strains of *Staphylococcus aureus* (Food Research Institute culture collection) which produced the following enterotoxins were chosen: FRI-100 (A^+), S-6 (A^+ B^+), and S-6R (A^+ B^-). These strains were maintained in dried form on porcelain beads by the method of Hunt et al. (7).

Inocula. Inocula were prepared by putting beads of the strains into broths (50 ml in 250-ml Erlenmeyer flasks) of 4% PHP, a pancreatic digest of casein (Mead Johnson International, Evansville, Ind.). These pH 7.0 broths were supplemented with 0.05 g of thiamine per liter and 1.0 g of niacin per liter. The cultures were incubated at 37 C for 12 to 18 h on a gyratory shaker (280 rpm) and then diluted in 0.1% peptone (Difco) blanks, so that the inoculum was 1 to 10 colony-forming units (CFU)/ml of broth.

Media. Growth and toxin synthesis were studied in 2-liter Erlenmeyer flasks containing 600 ml of 0.5, 1.0, 2.0, 3.0, 4.0, 6.0, or 8.0% PHP broths. The vitamin concentrations were adjusted proportionately to the concentrations in the 4% PHP broths used to prepare inocula. Also, broths (4% PHP) containing yeast extract (Difco) at final concentrations of 0.5 and 1.0% were used. A standard broth (50 ml in 250-ml Erlenmeyer flasks or 600 ml in 2-liter Erlenmeyer flasks) of 3% PHP plus 3% N-Z Amine NAK (a pancreatic digest of casein; Sheffield Chemical Co., Norwich, N.Y.) fortified with the vitamin concentrations given above for 4% PHP was used.

Sterile solutions of the following substrates (measured in percentages) were added to 50-ml sterile 0.5% PHP broths in 250-ml Erlenmeyer flasks either prior

to inoculation or after the optical density (OD) of the cultures (diluted 1:10) had become 0.065, 0.10, 0.20, and 0.30 to effect the concentrations listed: glucose (0.1, 0.2, 0.5); sodium pyruvate (0.1, 0.5); fumarate (0.01, 0.1); sodium oxalacetate (0.01, 0.1); L-glutamate (0.02, 0.1); L-arginine monochloride (0.02, 0.1); and yeast extract (0.2, 0.5).

Triplicate platings were made for appropriate dilutions on brain heart infusion agar (Fisher).

Phosphate-buffered saline (PBS) was prepared by adding enough 0.02 M KH_2PO_4 in 0.9% NaCl to 0.02 M Na_2HPO_4 in 0.9% NaCl so that the pH became 7.4. Some solutions of PBS were heat sterilized; to others, merthiolate (thimerosal; Sigma Chemical Co.) was added at a final concentration of 0.01%.

Physiological characteristics of strains S-6, S-6R, and FRI-100. The three strains were grown in 50 ml of 4% PHP broths for 18 h and then diluted and plated so there would be 60 to 90 CFU/plate. After 24 h of incubation at 37 C, three plates of the appropriate dilution were replica plated onto the following agars: APT agar (Difco) plus 0.02% NaN_3; egg-yolk tellurite agar (1); coagulase agar (Difco) with 7.5% human plasma (Warner-Chilcott); phosphatase agar (2); deoxyribonuclease (DNase) agar (Difco); glucose agar (purple broth base agar; Difco) that contained 1.0% glucose; mannitol agar (phenol red base agar; Difco) that contained 1.0% mannitol; and anti-enterotoxin serum agar (4% PHP broth, 1.5% Noble agar [Difco], and enough filter-sterilized antiserum to effect a titer of 1 [20]). After 18 h of incubation at 37 C, the plates were treated with the appropriate test reagents.

Four tubes of 1.0% glucose in purple broth base and 1.0% mannitol in phenol red base were inoculated with 0.05 ml of 18-h cultures. Duplicate tubes of glucose and mannitol broths were layered with paraffin to achieve anaerobiosis. The tubes were read 24 and 48 h after inoculation.

Growth studies. Cultures were incubated on a gyratory shaker (280 rpm) at 37 C. A desk-top gyratory shaker was placed in constant-temperature incubators for the studies at 26 and 30 C. At certain intervals, 1.0 ml of culture was aseptically withdrawn and diluted for surface plates which were made in triplicate and incubated at 37 C for 18 to 24 h.

The pH and OD were determined at the time that samples were plated. The OD was read on a Coleman nepho-colorimeter at 655 nm and also on a Beckman spectrophotometer (model DB-G) at 655 nm.

For the analysis of growth and toxin during the exponential growth phase, eight broths (toxin broths) and a control broth were inoculated. Samples of various volumes were withdrawn after 6 to 12 h of incubation. Surface plates were made at hourly intervals of both the control broth, in which growth was continually followed, and a toxin broth, in which the toxin concentration and growth were determined. The toxin broth, after having been sampled, was discarded. The control broth, sampled (1.0 ml), was returned to the shaker. These procedures were repeated until the last toxin broth had been sampled. After this, 5 ml was withdrawn from the control broth at hourly intervals for plate counts and toxin analysis by single gel diffusion. One set of toxin broths of strains S-6 and S-6R was incubated for 34 to 36 h and later tested for enterotoxins. The toxin broths were concentrated as described below.

Growth parameters for S. aureus S-6. Samples (10 ml) were centrifuged for 10 min at 17,000 $\times$ *g* at −4 C. The supernatant fluids were decanted and placed on single gel diffusion tubes, after the addition of 0.1 ml of 1% merthiolate.

The pellet was washed twice in 10 ml of PBS and resuspended in 5.0 ml of PBS. Two samples (1.5 ml) were pipetted into weighed aluminum pans, which were placed in an oven (102 C) for 36 h and then in desiccators (P_2O_5) for 8 h. The pans were weighed and the dry weights were calculated.

Two samples (1.0 ml) were dispensed into test tubes and frozen for later analysis of ribonucleic acid (RNA) and protein. RNA samples were digested with 0.15 ml of 60% perchloric acid at 70 C for 30 min, cooled, and centrifuged for 10 min at 10,000 rpm. Cellular RNA was quantitated by the method described by Schneider (17). Solutions of yeast RNA (Difco) were used as standards.

Protein samples (1.0 ml) were digested in NaOH (1.0 ml) at 80 C for 30 min. The concentrations of NaOH used depended on the concentration of protein in the samples. Various dilutions of these digests were made, and the concentration of NaOH in these diluted samples was 0.025 to 0.05 N. A modified Lowry method (9), employing 1% sodium citrate instead of 1% sodium-potassium tartrate, was followed to assay for cellular protein. Casein Hammersten (Nutritional Biochemicals Corp.) was used as the standard.

Enterotoxin analysis. (i) Single gel diffusion tube method. Enterotoxin was quantitated on single gel diffusion tubes as described by Kato et al. (8). Concentrates for gel diffusion analysis were prepared by dialyzing known volumes of supernatant fluids against 50% (wt/vol) Carbowax 20 M (polyethylene glycol) for 24 h. The enterotoxin was rinsed from the tubing with a minimum amount of buffer (0.01 M Na_2HPO_4, pH 7.4), lyophilized, and resuspended in distilled water.

(ii) Microslide technique. By the double gel diffusion slide method described by Casman and Bennett (3), controls showed that an estimated 50 to 60% of the enterotoxin in a 100-fold concentrate was actually detected. Our results from microslides were useful as general estimates.

Concentrates for the microslide and reverse passive hemagglutination were prepared in the following way. The supernatant fluids were passed through a Gelman metricel filter (pore diameter 0.20 μm), dialyzed against Carbowax for 24 h, removed from the dialysis tubing with buffer, dialyzed in cold distilled water for 48 h, dialyzed against Carbowax for 24 h, centrifuged at 0 C for 60 min at 1500 rpm in tapered glass tubes, and lyophilized. The lyophilized powders were reconstituted with distilled water to effect the desired concentrations.

(iii) RPHA. The reverse passive hemagglutination

(RPHA) method was used as described by Silverman et al. (18). Enterotoxin antibodies were adsorbed on sheep red blood cells treated with tannic acid (1:10,000). Sensitizing buffer (pH 7.4) contained 0.0075 M Na_2HPO_4-KH_2PO_4 and 0.45% NaCl. Normal rabbit serum (1%) was added to sensitizing buffer (pH 7.3). Controls showed that 95% of the known concentrations of toxin could be detected.

Statistical analysis. The exponential growth rates (k) and the exponential rates of enterotoxin synthesis were determined by least squares. In this paper, the corresponding generation time (g) is parenthetically expressed in minutes after k is given. The time in which the enterotoxin concentration doubled is parenthetically expressed in minutes after the rate of enterotoxin synthesis is given.

RESULTS

Growth and enterotoxin B synthesis by S. aureus S-6 in 4% PHP broth. The growth of *S. aureus* S-6 was followed for 24 h in 600 ml of 4% PHP broth to determine when enterotoxin B could be detected by the single gel diffusion tube method. By this method, enterotoxin B was not detected until 12 h, a time which coincided with the end of the exponential growth phase (Fig. 1). The level of enterotoxin B at 12 h was 1 to 2 μg/ml, which is the limit of the single gel diffusion tube method.

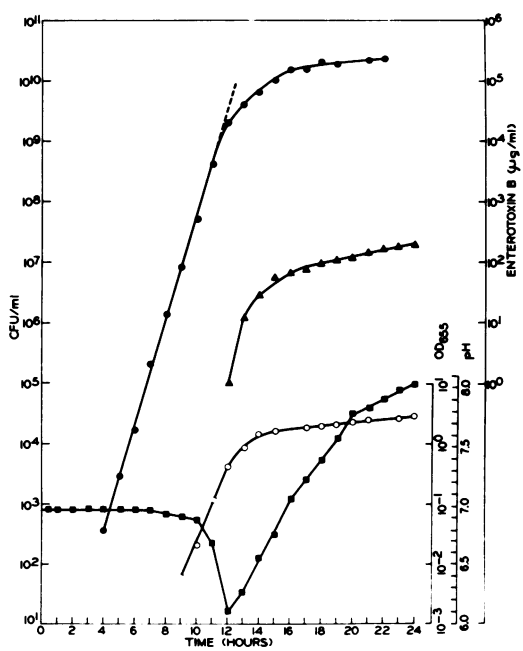

FIG. 1. *Rate of enterotoxin B synthesis (results by single gel diffusion tubes) and rate of growth in 4% PHP broth by S. aureus S-6. Symbols:* ●, *CFU/ml;* ○, *optical density;* ■, *pH;* ▲, *enterotoxin B.*

Growth and toxin synthesis in broths with various concentrations of PHP. The rates of growth and enterotoxin B synthesis were studied between 9 and 18 h in broths containing different concentrations of PHP. From Fig. 1 it was found that this time interval included part of the exponential phase and the transitional phases thereafter.

Table 1 shows that the exponential growth rates were independent of the concentration of PHP. Regardless of medium, toxin was always detected very soon after the growth rate had begun to decrease. At that time, there were between 10^9 to 2×10^9 CFU/ml.

The maximal rates of toxin synthesis were dependent on the concentration of PHP in the broths, but not directly proportional to it (Fig. 2). The maximal rate, however, was generally preceded by a different rate. Diphasic curves became more apparent as the concentration of PHP was increased. Like the previous studies, the concentration of toxin as determined by the single gel diffusion tube method was 1 to 2 μg/ml. The level of enterotoxin B could not be increased beyond 1 to 2 μg/ml prior to the end of the exponential growth phase by increased concentrations of PHP.

Effect of other substrates on growth and enterotoxin B synthesis. When 4% PHP broth was fortified with 0.5 or 1.0% yeast extract prior to the end of the exponential growth phase, the growth began to decrease as the population approached 10^9 to 2×10^9 CFU/ml with toxin detected at that time. Similar results were observed when various concentrations of glucose, pyruvate, fumarate, oxalacetate, arginine, glutamate, and yeast extract were added to 0.5% PHP broths either prior to inoculation or 1 to 2 h before growth was expected to decrease. Added nutrients, therefore, did not alter the relationships which had been previously observed. Again, the level of enterotoxin B could not be increased beyond 1 to 2 μg/ml either prior to or at the end of the exponential phase of growth.

Growth and enterotoxin B synthesis. The relationship of enterotoxin B synthesis to growth was studied under conditions that retarded growth. When *S. aureus* S-6 was incubated at 26 C, the exponential growth rate was 1.0 h^{-1}. Growth proceeded at this rate for 40 h. The rate then slightly decreased and remained constant till at least 44 h. The level of enterotoxin B reached 1 to 2 μg/ml after the growth rate had begun to decrease (40 to 41 h). The levels of cellular RNA, cellular protein, and enterotoxin B were quantitated to determine if

TABLE 1. *Effect of the PHP concentration on growth and enterotoxin B synthesis of S. aureus S-6*

PHP (%)	Growth		Stage of growth at time of toxin detection[a]				Enterotoxin synthesis, maximum rate (μg/ml/h)
	k (h^{-1})	k (h)[b]	Time (h)	Toxin (μg/ml)	CFU × 10^9/ml	pH	
0.5	2.5	14	15	0.9	1.8	6.64[c]	3.0
1.0	2.6	12	13	0.9	1.9	6.76[c]	5.5
2.0	2.6	11	12	1.0	1.6	6.30	8.5
3.0	2.5	12.25	12.25	10.4	3.3	6.35	11.5
3.0[d]		11.5	11.5	1.0	1.3		
4.0	2.6	12	12	1.0	1.0	6.10	17.0
6.0	2.7	12.25	12.25	10.5	4.0	6.27	18.0
6.0[d]		11.5	11.5	1.0	1.2		
8.0	2.6	15	15	1.7	1.1	6.45	22.5

[a] Enterotoxin B detected on single gel diffusion tubes.
[b] Time when growth rate is no longer exponential.
[c] pH is decreasing.
[d] Extrapolated values.

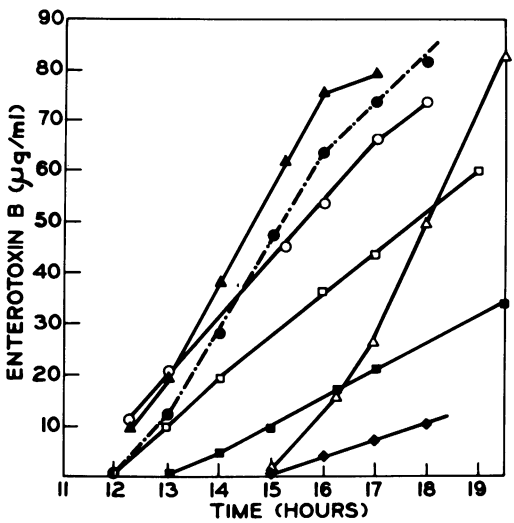

FIG. 2. *Effect of PHP concentration on rates of enterotoxin B synthesis by S. aureus S-6. Symbols:* ◆, *0.5% PHP;* ■, *1.0% PHP;* □, *2.0% PHP;* ○, *3.0% PHP;* ●, *4.0% PHP;* ▲, *6.0% PHP;* △, *8.0% PHP.*

the rates of cellular protein and toxin synthesis were comparable. A similar study was made at 37 C.

At 26 C, 1.0 μg of toxin per ml had accumulated once the population reached 2.25 × 10^9 CFU/ml. The mass of these cells was 24 to 26% RNA and 60 to 68% protein. At 37 C, an estimated 0.7 μg of enterotoxin B per ml had accumulated when the culture reached 1.25 × 10^9 CFU/ml. The mass of these cells was 16 to 18% RNA and 72 to 76% protein. A plot of CFU/milliliter versus dry weight/milliliter showed that cells grown in the same broths at different temperatures had the same cell weights. The proportion of cellular protein to RNA and that of enterotoxin B to RNA, at a given temperature, paralleled each other to a certain degree (Fig. 3). This was more striking at 26 C than at 37 C.

Synthesis of enterotoxin B by S. aureus S-6 during exponential growth. The growth rate for strain S-6 was calculated from Fig. 4 to be 2.8 h^{-1} (22 min), which was consistent with the data of six separate experiments in these studies and the data reported above. This rate decreased between 11 and 12 h, when the population increased to 10^9 to 2 × 10^9 CFU/ml. Enterotoxin B synthesis during exponential growth (Fig. 4) was at an extremely rapid rate, 5.6 h^{-1} (11 min). The rate decreased between 12 and 13 h when the population increased from 2 × 10^9 to 4 × 10^9 CFU/ml and the toxin increased from 4 to 16 μg/ml.

Growth and enterotoxin A synthesis. Three staphylococcus strains, S-6 (A^+, B^+), S-6R (A^+, B^-), and FRI-100 (A^+), were used in these experiments. Strain S-6 produced a very small amount of enterotoxin A in addition to the large amounts of enterotoxin B. Strain S-6R was a mutant of strain S-6 which no longer synthesized enterotoxin B but was able to produce the same small quantities of A as strain S-6. *S. aureus* FRI-100 was one of the highest enterotoxin A producers available, producing 4 to 5 μg/ml.

The exponential growth rates of these three strains were different (Table 2). Strain S-6R grew slightly faster than strain S-6 and entered the transitional phase when the population had

reached the same cellular density observed for strain S-6. This population lowered the pH of the broth in a manner similar to that shown for strain S-6 (Fig. 1; Table 1). No enterotoxin B was detected in 1,000-fold concentrates of S-6R samples. In other ways, *S. aureus* S-6R was physiologically similar to strain S-6. Both reduced tellurite, fermented glucose and mannitol, and elaborated DNase, coagulase, lipase, and phosphatase.

Strain FRI-100 was able to grow on mannitol and glucose aerobically and anaerobically, to reduce tellurite, and to elaborate the exoenzymes given above; but, its growth rate was much slower than that of strains S-6 and S-6R

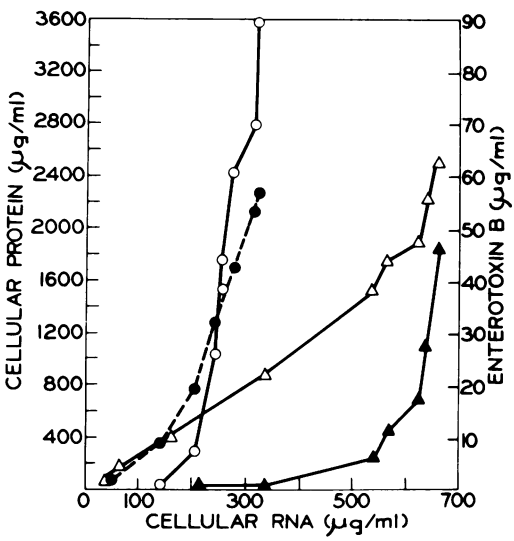

FIG. 3. *Comparison of enterotoxin B-RNA and cellular protein-RNA at 37 and 26 C. Symbols:* ○, *toxin B at 37 C;* ●, *cellular protein at 37 C;* ▲, *toxin B at 26 C;* △, *cellular protein at 26 C.*

and it did not produce enterotoxin B. Enterotoxin A was detectable by the single gel diffusion tube method when the population had reached 9×10^8 CFU/ml.

These three strains entered the transitional phase when the population was close to 10^9 CFU/ml. Once the population had become large enough, detectable levels of toxin (single gel diffusion tube method) were reached by the transitional phase of growth.

The exponential rate of enterotoxin A synthesis by strain S-6 was 2.6 h^{-1} (23 min), with a leveling between 11 and 12 h (Fig. 4). The population in this interval increased from 3.3×10^8 to 1.9×10^9 CFU/ml, and the concentration of enterotoxin A increased from 3.5×10^{-3} to 1.6×10^{-2} µg/ml. Whether enterotoxin A continued to increase could not be determined, since the concentration of enterotoxin A produced by strain S-6 is very low. Slight increases would not have been sufficiently different to be noticeable. The samples obtained from strain S-6R contained the same concentrations of enterotoxin A as the ones from strain S-6. Maximum yields of enterotoxin A after 36 h of incubation were 0.1 to 0.3 µg/ml.

Growth and enterotoxin A synthesis by S. aureus FRI-100. To study enterotoxin A synthesis under more optimum conditions, *S. aureus* FRI-100 was grown and assays for enterotoxin were made. *S. aureus* FRI-100 was found to have an exponential growth rate of 1.9 h^{-1} (32 min). This was consistent with the data of four separate growth studies. The rate decreased near the time (18.5 to 19.5 h) that 1 µg of toxin per ml had accumulated and the population had reached 10^9 CFU/ml (Fig. 5).

The exponential rate of enterotoxin A synthesis was 1.8 h^{-1} (32 min). Enterotoxin A was synthesized by *S. aureus* FRI-100 at a rate equal

TABLE 2. *Comparison of growth and enterotoxin synthesis by S. aureus strains S-6, S-6R, and FRI-100 in broths of 4% PHP*

Strain	Growth			Stage of growth at time of toxin detection[a]			
	k (h^{-1})	*g* (min)	*k* (h)[b]	Time (h)	Toxin (µg/ml)	CFU × 10^8/ml	pH
S-6	2.7	22	12	12	1.0[c]	1.0	6.12
S-6R	3.1	19	11		0.0[c]	1.4	6.31
FRI-100	1.9	31	20	20	1.1[d]	0.9	6.66[e]

[a] Enterotoxins detected by single gel diffusion tube method.
[b] Time when growth rate is no longer exponential.
[c] Concentration of enterotoxin B; enterotoxin A is not detectable by single gel diffusion for strains S-6 and S-6R.
[d] Concentration of enterotoxin A.
[e] pH decreases to at least 6.40 in next hour; then it increases.

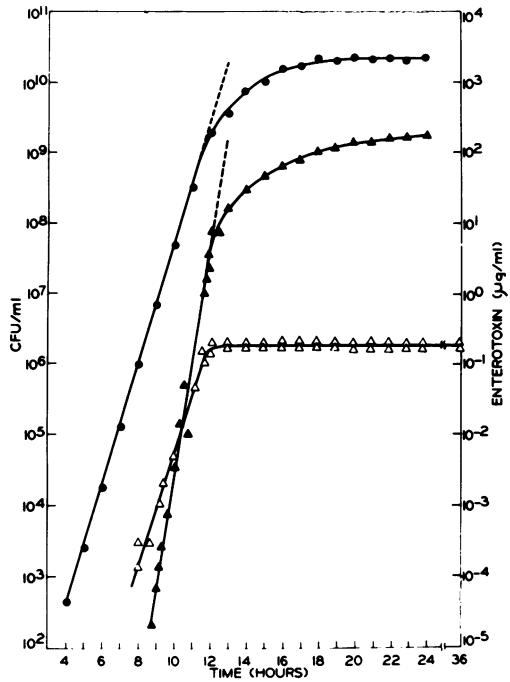

FIG. 4. *Rates of growth and enterotoxins A and B synthesis by S. aureus S-6. Symbols: ●, CFU/ml; △, enterotoxin A; ▲, enterotoxin B.*

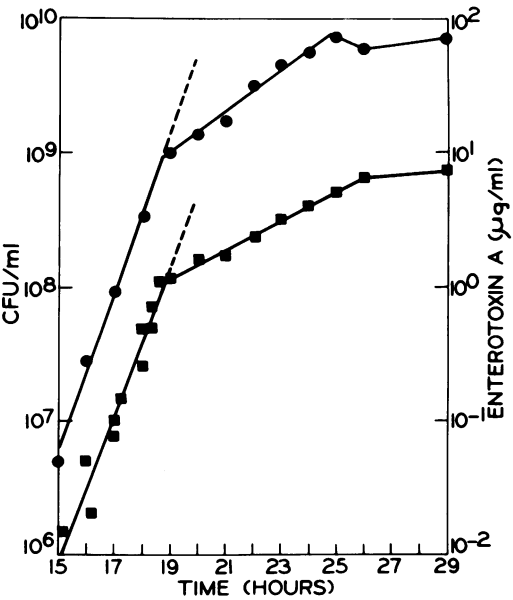

FIG. 5. *Rates of growth and enterotoxin A synthesis by S. aureus FRI-100. Symbols: ●, CFU/ml; ■, enterotoxin A.*

to the growth rate; this was like the relationship of enterotoxin A synthesis to growth seen with *S. aureus* S-6. Because *S. aureus* FRI-100 produced higher levels of enterotoxin A than strain S-6, incremental changes were easier to observe. Although the rate of toxin synthesis was markedly reduced at the time that growth decreased (Fig. 5), enterotoxin A continued to be synthesized during transitional growth at a rate commensurable with growth.

DISCUSSION

The relationship of enterotoxin synthesis to growth has been clarified. Enterotoxins A and B are both produced during all phases of growth. Marked differences are seen in the rates at which enterotoxins A and B are synthesized. These account for the high concentrations of enterotoxin B and the correspondingly lower levels of enterotoxin A that are detectable under comparable conditions.

The data presented here concerning enterotoxin B synthesis conflict with previous reports. Markus and Silverman (11) reported that enterotoxin B was produced at the end of the exponential phase of growth, and complementary reports (13, 14) claimed that enterotoxin B was derepressed at the end of the exponential phase of growth. These reports, however, were based on results obtained exclusively by a single gel diffusion tube method with 1 μg of enterotoxin per ml as the minimum detectable.

The fact that the exponential phase always ends when the population approaches 10^9 to 2×10^9 CFU/ml, regardless of medium or cultural strain, indicates that a population of this size either depleted essential nutrients or reached saturating conditions (16). Presumably, if nutrients had been exhausted by growth, subsequent addition of various substrates should have allowed rapid growth to continue beyond 10^9 CFU/ml. This was never observed. Regardless of when substrates were added, the growth rate decreased once the population had reached this density.

Other investigators (4, 11-13) have introduced the terms primary and secondary metabolites to enterotoxins A and B synthesis without consistent explanation of this terminology (4, 12). We believe these terms can be confusing and misleading, primarily because there is no one acceptable definition for them. We prefer to relate the enterotoxin production to growth of the organism. Our studies indicate that the synthesis of enterotoxin B is a function of RNA in a manner similar to the synthesis of cellular protein. These studies, along with those show-

ing the rates of enterotoxin B synthesis during the late exponential and early transitional phases of growth, support the data obtained with RPHA. These data show that enterotoxin A and B are produced during all stages of growth.

ACKNOWLEDGMENTS

This research was supported by the College of Agricultural and Life Sciences, University of Wisconsin, Madison, and by Public Health Service grant AI-07615 from the National Institute of Allergy and Infectious Diseases.

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