INFECTION AND IMMUNITY, Mar. 1977, p. 781-788

Vol. 15, No. 3
Printed in U.S.A.

Nutrition and Enterotoxin Synthesis by Enterotoxigenic Strains of *Escherichia coli*: Defined Medium for Production of Heat-Stable Enterotoxin

JOHN F. ALDERETE and DONALD C. ROBERTSON*

Department of Microbiology, University of Kansas, Lawrence, Kansas 66045

Received for publication 16 November 1976

A defined medium has been developed which supports synthesis of heat-stable enterotoxin (ST) by porcine and bovine strains of enterotoxigenic (ENT^+) *Escherichia coli* in levels equivalent or better than a complex Casamino Acids-salts medium. The medium components did not support production of heat-labile enterotoxin (LT) but were similar for ST synthesis by ENT^+ strains producing only ST and those which produced ST in addition to LT. The amino acids in Casamino Acids found to be necessary for growth and enterotoxin synthesis were proline, serine, aspartic acid, and alanine. Maximal growth and toxin levels were obtained after 8 h of incubation. Improved growth, but not an increase in synthesis of ST, was observed in the presence of Mg^{2+}, Mn^{2+}, and Fe^{3+} compared with Mg^{2+} alone. A chelator, tricine, was necessary for maximal cell densities, probably to solubilize trace ions and make them more available to the bacteria. Increased growth was observed upon addition of glucose to both complex and defined media; however, glucose as well as gluconate and pyruvate appeared to cause repression of toxin synthesis. Addition of vitamins, oleic acid, or DL-lactic acid to the defined medium slightly increased levels of ST.

Enterotoxigenic strains of *Escherichia coli* colonize the small intestine of humans and neonatal animals (pigs, calves, and lambs) and elaborate two distinct extracellular products called enterotoxins, each of which is capable of causing a cholera-like illness (9, 16, 23, 26–28). One enterotoxin is of high molecular weight and heat labile, and appears to be a protein, whereas the other is of low molecular weight and heat stable (9, 26–28). The high-molecular-weight enterotoxin has been partially purified and characterized (5, 25), but purification of the heat-stable toxin (ST) from enterotoxigenic *E. coli* grown either in the complex medium of Evans et al. (7) or Trypticase soy broth has been difficult, partially because of the complexity of the medium. Also, high-molecular-weight medium components may associate with the enterotoxins and complicate purification and isolation. We wished to develop a minimal defined medium with growth and toxin levels equivalent to a complex medium containing Casamino Acids (CAA) and yeast extract (YE) in order to facilitate purification and studies on the mode of action of the low-molecular-weight ST. This report describes the development of such a defined medium and the effect of various nutritional parameters on enterotoxin synthesis.

MATERIALS AND METHODS

Bacterial strains. The *E. coli* strains of porcine and bovine origin (ENT^+) used in this study were supplied by Harley Moon of the National Animal Disease Center, Ames, Iowa, and included five strains that produce only heat-stable enterotoxin (ST) and one strain that produces both heat-labile enterotoxin (LT) and ST (Table 1). A non-enterotoxigenic *E. coli* strain O111B4 was obtained from Clarence S. Buller of this department.

Conditions for growth and enterotoxin production. Cultures were grown aerobically in 250-ml-capacity Erlenmeyer flasks containing 50 ml of medium. The flasks were incubated at 37°C on a New Brunswick rotary shaker (300 rpm). The starter culture was grown in M-9 minimal medium (18) for 8 h, and each experimental flask was inoculated with the starter culture using a volume to give an initial absorbancy of 0.05 (620 nm). The initial pH was 7.5, and samples were withdrawn at hourly intervals for measurement of pH, enterotoxin, and growth. The samples removed were small enough so that the total volume was not decreased significantly. Cells were removed by centrifugation in sterile centrifuge tubes, at 23,500 × *g* for 30 min, and culture supernatants were stored at −20 or −70°C until assay. No loss of toxin activity due to freezing was observed.

Preparation of media. The complex medium of CAA and YE was prepared as described by Evans et al. (7) except that the pH of the medium was adjusted to 7.5 with 5 N NaOH.

TABLE 1. *Escherichia coli strains used in nutrition studies*

Strain	Source	Toxin produced[a]
O111B4		0
431	Porcine	ST
987	Porcine	ST
1413(74-5208)	Porcine	ST
1261(2176E8)	Porcine	ST
1417(B41)	Bovine	ST
1362	Porcine	ST-LT

[a] 0, No toxin; ST, heat-stable toxin; ST-LT, heat-stable and heat-labile toxins.

The defined medium described in Table 7 was prepared by making a twofold stock solution of basal salts and trace salts and a 10-fold stock solution of the amino acids so that the final concentrations after dilution were those listed in Table 7. Both solutions were adjusted to pH 7.5 before autoclaving. The stock solutions were added to sterile flasks, and sterile distilled water was added to a final volume of 50 ml. Complete medium contained all 18 amino acids at concentrations equivalent to those present in a 2% CAA solution (19). The amino acids for the complete medium were prepared as a 10-fold stock solution excluding cystine, which was dissolved separately at a concentration of 2 mg/ml and used at a final concentration of 100 μg/ml. Each medium contained the trace ions, salts, and tricine listed in Table 7 unless otherwise stated.

Toxin assay. Swiss albino mice (3 to 5 days old) were taken from their mothers and randomly divided into groups of three. Samples containing ST were assayed by making serial twofold dilutions in sterile phosphate-buffered saline, and 0.1 ml of undiluted supernatant or diluted supernatant containing Evan's blue was injected intragastrically with 25-gauge tubing attached to a 1-ml tuberculin syringe. Intestinal fluid accumulation was measured after 3 h of incubation at 25°C. Animals were sacrificed by surgical dislocation, a midline incision was made in the abdomen, and the intestine was removed with forceps. The intestines and carcasses from three animals were pooled, and the weight of the intestines was compared with the remaining body weight, hereafter referred to as the gut-to-body-weight (G/B) ratio. The assay was reliable and reproducible as has been found by others (8, 10). A ratio of less than 0.065 was considered negative, those between 0.065 and 0.09 were questionable, and a ratio of over 0.09 was strongly positive. The maximal G/B ratio ranged between animals from 0.14 to 0.17; hence, multiple dilutions were assayed to observe the dose response for each sample. Each experimental sample was assayed using three mice, and each experiment was done in triplicate. The dilution that gave a response greater than 0.09 was quite reproducible and could be used to express enterotoxin levels in different media under varying growth conditions. Non-enterotoxigenic *E. coli* grown under the same conditions as ENT^+ strains was used as a negative control. Animals with no dye in the intestine at autopsy were discarded.

Reagents used. All L-amino acids were dissolved in 0.01 N HCl and adjusted to pH 7.5 with 5 N NaOH. 2,3-Dihydroxybenzoic acid and *p*-hydroxybenzoic acid were prepared as a 1,000-fold concentrated solution and incorporated into the final medium at 10^{-5} M. Stock solutions of 1 M morpholinopropane sulfonate (MOPS) were adjusted to pH 7.5, filter sterilized, and stored at −20°C until use. All sugars were used as 25% stock solutions (wt/vol). The sodium salts of fatty acids were dissolved in 4% polyethylene (20) cetyl ether-Brij 58. All vitamins were combined as a 100-fold working solution, sterilized by filtration, and used at the final concentrations as follows: *p*-aminobenzoic acid (0.10 μg/ml), pantothenate (0.20 μg/ml), thiamine (1.0 μg/ml), riboflavin (0.25 μg/ml), folic acid (0.01 μg/ml), pyridoxine (1.0 μg/ml), niacin (0.40 μg/ml), and biotin (0.001 μg/ml).

All media components were purchased from Sigma Chemical Co. unless otherwise indicated. Brij 58 was obtained from Atlas Chemical Industries Inc., Wilmington, Del. 2,3-Dihydroxybenzoic and *p*-hydroxybenzoic acids were products of Aldrich Chemical Co., Milwaukee, Wis. CAA and YE were obtained from Difco Laboratories.

RESULTS

Properties of strains used for nutritional studies. The properties of the strains of *E. coli* shown in Table 1 have been documented in other laboratories and were screened using both the suckling mouse assay to detect ST (8, 10) and the Y-1 adrenal tumor cell assay, which is specific for LT (23). All strains except 1362 were positive only in the suckling mouse assay. Strain 1362 was positive in both assays and strain O11 B4 was negative in both. Aliquots of all culture supernatants were heated at 65°C for 30 min to confirm the presence of ST activity.

Growth and toxin synthesis in complex medium. The complex media of Evans et al. (7), containing CAA and YE, Trypticase soy, and brain heart infusion broth, were compared in preliminary experiments for their ability to support growth and enterotoxin synthesis (data not shown). The complex medium of CAA and YE supported growth and toxin synthesis equivalent to or better than the other complex media and was chosen as a starting medium because each of the components could be tested for its contribution to growth and toxin production. All strains showed maximal cell densities by 18 h when grown in a CAA medium in the presence or absence of YE. Cell densities were slightly less at 8 h, with optical densities (ODs) ranging from 5.5 to 6.5 in the absence of YE and ODs of 6.5 to 7.5 in the presence of YE (Table 2). Maximal enterotoxin levels in supernatants of *E. coli* that produced either ST or ST-LT were observed by 8 h; consequently, all assays were done at this time. YE did not appear to be

required by either strain for enterotoxin synthesis since the last dilution to give a positive response was 1:9 (1362) and 1:16 (431), respectively, for the supernatants of cells grown in CAA in the presence or absence of YE. The role of trace salts (TS) containing Mg^{2+}, Mn^{2+}, and Fe^{3+} in the complex medium was examined (Fig. 1). Elimination of TS from the complex medium resulted in poor growth, but addition of Mg^{2+} resulted in growth equivalent to the TS. Since divalent cations are present as contaminants in CAA (20), we decided to examine the role of these ions later by using a defined medium. The other enterotoxigenic strains in Table 1 gave data similar to those obtained for strains 431 and 1362. Furthermore, unless otherwise stated, the data obtained with other strains were similar to strains 431 and 1362.

TABLE 2. *Heat-stable enterotoxin production by ENT+ E. coli strain 431 and strain 1362 in complex media*

Strain	Growth medium[a]	Incubation time (h)	Optical density (620 nm)	Toxin activity
431	CAA	8	5.4	1:16
	CAA	18	8.5	1:16
	CAA-YE	8	7.3	1:16
	CAA-YE	18	9.0	1:16
1362	CAA	8	6.5	1:8
	CAA	18	7.0	1:8
	CAA-YE	8	7.0	1:8
	CAA-YE	18	7.5	1:8

[a] Casamino Acids (CAA), 2%; yeast extract (YE), 0.6%; plus trace salts.

[b] Toxin activity, Twofold serially diluted supernatant in sterile PBS, which gave a G/B ratio ≥ 0.090.

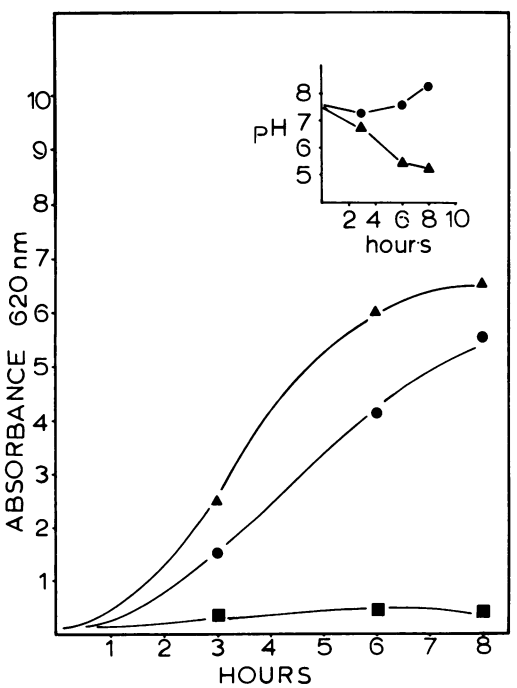

FIG. 1. *Growth and pH by ENT+ E. coli strains 431 and 1362 in 2% CAA (●), CAA supplemented with 1% glucose (▲), CAA in the absence of Mg^{2+} (■).*

Higher levels of enterotoxin might be observed with increased growth; therefore, we attempted to supplement CAA with growth factors present in YE. However, enterotoxin levels were not increased by addition of vitamins, purines, and pyrimidines. Addition of glucose (1%) did increase growth (Fig. 1), but ST was not detectable in the supernatant using the suckling mouse assay (Table 3). A G/B ratio less than 0.090 was observed for the undiluted supernatant of strain 431 grown in CAA supplemented with glucose compared with the last positive dilution of 1:16, where glucose was omitted. The decreased toxin synthesis may be due to repression of enterotoxin synthesis by glucose (4) or the release of ST may be inhibited by low pH, as has been found for *Vibrio cholerae* (3). An effective means of buffering the pH must be found in order to distinguish between the two possible mechanisms.

TABLE 3. *Heat-stable enterotoxin synthesis by enterotoxigenic E. coli strain 431 in different growth media*

Medium	Optical density (620 nm)	pH	Enterotoxin level[a]
Complex[b]			
CAA	5.4	8.4	1:16
CAA + YE	7.3	8.4	1:16
CAA + 1% glucose	6.5	5.4	ND
Complete[c]			
No additions	4.0	8.0	1:16
+ 1% Glucose	4.5	5.2	ND
+ 1% Glucose + MOPS	5.7	5.7	ND
Defined[d]			
No additions	3.5	7.7	1:16
+ 0.25% Glucose	4.2	7.1	ND
+ 0.25% Glucose + MOPS[e]	4.8	7.7	UND
+ 1% Glucose	4.8	5.2	ND

[a] Defined in Table 2 with 8 h of incubation. ND, Not detected; UND, undiluted.

[b] Concentrations are as in Table 2.

[c] Medium with 18 amino acids present at concentrations equivalent to a 2% CAA except for cystine, which is present at 100 μg/ml.

[d] Medium consisting of the four amino acids listed in Table 7. The concentrations are equivalent to 2% CAA.

[e] MOPS, Morpholinopropane sulfonate.

Amino acids required for growth and toxin synthesis. The objective was to identify those amino acids present in CAA necessary for growth and enterotoxin synthesis. All strains exhibited moderate growth (OD, 3.5) in complete medium containing L-amino acids at levels equivalent to 2% CAA and growth could not be increased by addition of purines, pyrimidines, and vitamins. Enterotoxin levels were similar in the supernatant of the equivalent (complete) medium compared with supernatants from strain 431 grown in 2% CAA (Table 3), where a 1:16 dilution of either supernatant gave a G/B ratio greater than or equal to 0.090. The pH remained above 7.0 in the media where glucose was not present (Fig. 2 and Table 3). Addition of MOPS to control pH in the presence of glucose did not increase enterotoxin levels.

Since interactions between amino acids may be both stimulatory and inhibitory, the amino acids were grouped as described by Callahan et al. (3) to establish the amino acids required for toxin synthesis. Only four mixtures of amino acids (3, 4, 8, 9) supported bacterial growth at a cell density greater than an OD of 1.0 after 18 h of incubation (Table 4). Little growth was observed at 8 h, which suggested that conditions were not optimal in any one mixture. Although toxin could be detected in undiluted aliquots of mixtures 2 and 4, mixtures 8 and 9 showed toxin levels equivalent to or slightly less than the complete medium. The important amino acids appeared to be in mixtures 8 and 9 since groups 2 and 4 each had one amino acid common to mixture 8, that is, serine and aspartic acid, respectively. Combination of mixtures 8 and 9 and withdrawing amino acids one at a time showed that isoleucine, lysine, and arginine were not required for growth and enterotoxin synthesis and that proline, serine, aspartic acid, and alanine were required. Glutamic acid could be substituted for proline and arginine, but with decreased growth which may be due to competition between glutamic acid and aspartic acid for a common transport carrier. Comparable growth was observed in 8 h in the defined medium and the 2% CAA equivalent medium (Fig. 2), that is, OD of 3.5 versus 4.0. The defined medium produced ST levels by strains 431 and 1362 to the same dilution as the complex medium of CAA and YE and the complete medium (Table 3).

Effects of glucose on ST levels. The levels of ST for cells grown in either a complete or de-

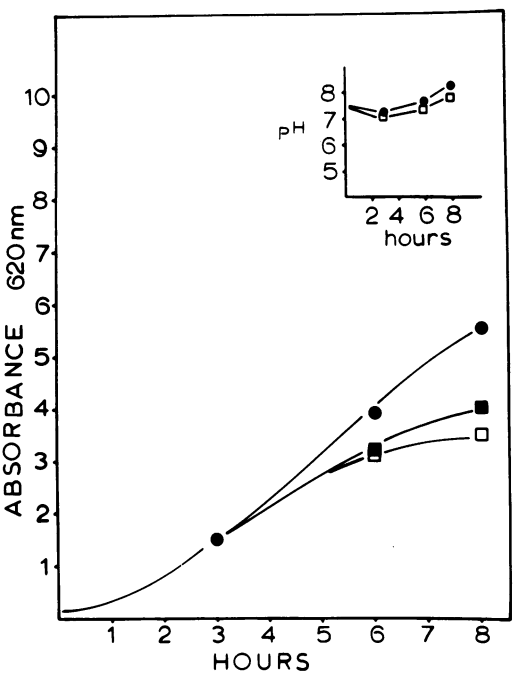

FIG. 2. *Representative growth and pH of the ENT⁺ E. coli listed in Table 1 grown in 2% CAA (●), complete (■), and defined (□) media.*

TABLE 4. *Effect of various amino acid mixtures on growth and heat-stable enterotoxin synthesis by ENT⁺ E. coli strain 431*

Mixture (OD_{620}, toxin)[a]	Amino acid mixture			
	1 (0.40, ND)[a,b]	2 (1.30, UND)[a,b]	3 (0.70, ND)[a,b]	4 (0.90, UND)[a,b]
5 (0.31, ND)	Histidine	Glycine	Cystine	Methionine
6 (0.55, ND)	Phenylalanine	Leucine	Isoleucine	Valine
7 (0.80, ND)	Glutamic acid	Tyrosine	Tryptophan	Threonine
8 (2.20, 1:8)	Arginine	Serine	Alanine	Aspartic acid
9 (3.20, 1:8)	Proline	Lysine	Alanine	Isoleucine
10 (4.3, 1:16) Complete medium[c]				
11 (6.8, 1:16) Complex medium[c]				

[a] Enterotoxin response determined as in Table 2.
[b] ND, Not detectable; UND, undiluted supernatant.
[c] Components and concentrations as described in Table 2.

fined medium were reduced by addition of glucose as a carbon source (Table 3). The low level of ST may be a function of pH as has been observed with *V. cholerae* (2) or catabolic repression by glucose (4). MOPS, an effective buffer (18), was added to either the complete medium or the defined medium with glucose; however, the pH increased only from 5.2 to 5.7 and 7.1 to 7.7, respectively. Decreasing the concentration of glucose resulted in less acid production, and in the presence of MOPS the pH was identical to the defined medium in the absence of glucose (pH 7.7). Enterotoxin was observed only in the undiluted supernatant, indicating a low level in the presence of glucose. Adjustment of pH or osmotic shocking of the bacteria by the procedure of Nossal and Heppel (21) at different stages of growth did not result in detectable ST levels (data not shown). Furthermore, release of lipopolysaccharide (LPS) by the procedure of Leive (14, 15) demonstrated that ST was not associated with the outer membrane of ENT+ *E. coli*. These data seem to rule out any pH effect on the release of ST or association of the low-molecular-weight enterotoxin with cellular macromolecules.

The effect of different growth factors and carbon sources on growth and enterotoxin synthesis in the defined medium was examined. Addition of vitamins resulted in a slight increase in toxin activity, where a 1:32 dilution of the supernatant was the last dilution to give a positive response (Table 5). Even though moderate growth was observed with M-9 minimal medium buffered with MOPS, a 1:2 dilution gave the last positive G/B ratio. Addition of DL-lactic acid to the defined medium did slightly increase toxin levels (1:32 versus 1:16). A slight decrease in toxin levels was found with pyruvate, and gluconate inhibited the synthesis of ST as did glucose. Exogenous fatty acids could play a nutritional or physical role, especially in the presence of limiting carbon source(s). Oleic acid slightly increased ST levels when added to the defined medium (Table 5). The same preparations of fatty acids used for the experiments were injected into baby mice and gave negative results.

TABLE 5. *Effect of various growth factors on the synthesis of heat-stable enterotoxin by ENT+ E. coli strain 431*

Additions to defined medium	Enterotoxin level[a]
Carbon sources[b]	
Glucose	ND
Gluconate	ND
Lactate	1:32
Pyruvate	1:8
Fatty acids[c]	
Oleate	1:32
Palmitate	1:16
Stearate	1:16
Other growth factors[d]	
Purines + pyrimidines	1:16
Vitamins	1:32

[a] Determined as described in Table 2. ND, Not determined.

[b] Carbon sources were added to a final concentration of 1.0%.

[c] Fatty acid concentrations were 100 μg/ml.

[d] Final concentrations: adenine, guanine, thymine, cytosine, and uracil were 0.2 mM. Each vitamin concentration is given in Materials and Methods.

Role of trace salts. The contribution(s) of divalent cations to growth and enterotoxin synthesis was examined to establish the role of the ions present in the CAA and YE medium. Growth of strain 431 depended on the presence of Mg^{2+} in either the CAA or defined medium (Fig. 1). Addition of either Mn^{2+} or Fe^{3+} to Mg^{2+} did not increase growth; however, supplementation with both did lead to slightly better growth (OD of 3.1 versus 2.5) and increased toxin (1:16 dilution versus 1:8) (Table 6). It was equivocal whether Fe^{3+} had any effect in ST synthesis. Tricine increased ST levels in the presence of TS and probably reflects improved solubility and availability of the cations to the bacteria. 2,3-Dihydroxybenzoic acid and *p*-hydroxybenzoic acid, precursors to natural chelators in *E. coli*, enterochelins, had no effects on the synthesis of ST.

Because Fe^{3+} is an important factor in the

TABLE 6. *Effects of trace ions, benzoic acids, and tricine on heat-stable enterotoxin synthesis by ENT+ E. coli strain 431 in a defined medium*[a]

Additions	Optical density (620 nm)	Enterotoxin level[b]
Trace salts[c]		
No trace salts	0.30	ND
+ $MgSO_4$	2.5	1:8
+ $MgSO_4$ + $MnCl_2$	2.3	1:8
+ $MgSO_4$ + $FeCl_3$	2.6	18
+ All	3.1	1:16
Benzoic acids and tricine[d]		
+ Benzoic acids	2.7	1:16
+ Tricine	3.0	1:32

[a] Components are described in Table 7.

[b] Determined as described in Table 2.

[c] Trace salts were prepared as described by Evans et al. (7).

[d] 2,3-Dihydroxybenzoic acid, *p*-hydroxybenzoic acid, and tricine were prepared as described in Materials and Methods and added aseptically before addition of trace salts.

synthesis of other bacterial exotoxins and is difficult to remove from all reagents, we wanted to show that iron was not required for the synthesis of ST. Conalbumin, an iron-binding protein, was added to the defined medium in the absence of benzoic acids and tricine and in the presence or absence of exogenous Fe^{3+}. Removal of contaminating and exogenous Fe^{3+} from reagents by conalbumin had no effect on enterotoxin synthesis, while slightly decreasing growth as expected.

DISCUSSION

This study establishes the requirement for certain amino acids and examines the role of the different medium components on growth and toxin synthesis by enterotoxigenic *E. coli*. A complex medium of choice was chosen as a starting base because the role on growth and toxin synthesis by the different complex components could be ascertained. Relative toxin levels were monitored by doing twofold serial dilutions of the supernatant as opposed to simply assaying the supernatants for toxin presence as has been done by previous investigators. A medium was devised that incorporated the contributions of each component of the complex medium.

Growth in the defined medium (Table 7) is about one-half that of a complex CAA-YE medium, but gives similar levels of ST (Table 3). Salts in the defined medium were at concentrations found by Neidhardt et al. (18) to be optimal for enterobacteria. The cations Fe^{3+} and Mn^{2+} increased cell density in the presence of Mg^{2+} but only upon addition of tricine, a chelator that solubilizes the ions and keeps them readily available to the bacteria. Decreased growth by *V. cholerae* has been reported to be caused by insoluble complexes between amino acids and divalent cations (3), and the effect was reversed by nitrilotriacetic acid, another metal chelator. Neidhardt et al. (18) observed that benzoic acids, precursors to enterochelins, and tricine were important for good growth of the enterobacteria; however, the benzoic acids did not affect growth or toxin synthesis by ENT^+ strains of *E. coli*.

TABLE 7. *Defined medium for heat-stable toxin synthesis by enterotoxigenic Escherichia coli*

Component	Final concn ($\times 10^{-3}$ M)
NaCl	40.0
K_2HPO_4	50.0
NH_4Cl	18.0
Trace salts	
$MgSO_4$	0.203
$MnCl_2$	0.025
$FeCl_3$	0.030
Tricine	10.0
Amino acids	
Proline	12.37
Aspartic acid	6.57
Alanine	4.40
Serine	6.23

The amino acid combinations used by Callahan et al. (3) for the determination of required amino acids pointed to multiple requirements when compared with the complete medium since growth was poor at 8 h for all amino acid mixtures. Combination of mixtures 8 and 9 resulted in reasonable growth and good ST levels by 8 h. Since combination of mixtures 8 and 9 included the amino acids common between groups 2, 4, and 8, and since growth and toxin levels were comparable to a medium with all of the amino acids present in 2% CAA, it was then possible to eliminate those amino acids that were not required for either growth or toxin synthesis. Arginine, lysine, and isoleucine were found not to be required, whereas elimination of any of the remaining four amino acids (proline, aspartic acid, serine, and alanine) resulted in decreased growth. Glutamic acid could be used to substitute for proline, but growth was decreased. Addition of individual amino acids or combinations of amino acids to the four amino acids did not affect growth or toxin synthesis, suggesting that other amino acids were not required. The amino acids included in the defined medium were at concentrations equivalent to 2% CAA. Increasing the concentration of each amino acid while keeping the other three at the 2% CAA equivalent concentrations did not increase growth or levels of ST; however, decreasing the concentrations of each of the four amino acids to less than a 2% CAA equivalent concentration resulted in decreased growth (Fig. 2). The amino acids necessary for synthesis of *E. coli* enterotoxin are similar to those required for synthesis of cholera toxin by *V. cholerae*, except that proline was substituted for glutamic acid and arginine. However, since no carbon source other than the amino acids is provided, these data suggest that the amino acids are the source of energy as well as precursors of anabolic pathways.

The fact that toxin could not be detected with addition of glucose to various media or when glucose was used as the sole carbon source in M-9 minimal medium suggests that glucose represses the synthesis of ST. A pH effect like that observed by Callahan and Richardson (2), where an acid environment inhibited the release of enterotoxin but where the toxin could be detected after adjusting the pH, was not ob-

served with either ST- or LT-ST-producing *E. coli*. ST was not detected by adjustment of pH or osmotic shocking to remove the periplasmic proteins. Since it has been reported that LT is associated with LPS (5), we selectively released LPS from the outer membrane of *E. coli* as described by Leive (14, 15); however, ST activity was not found associated with LPS (data not shown). Gluconate also repressed toxin synthesis, and pyruvate slightly decreased levels of ST. Glucose, gluconate, and pyruvate repression have been observed for several inducible enzymes (4, 22).

Supplementing the defined medium with vitamins, lactate, or oleate slightly increased toxin activity. An explanation of these results is not available; however, each addition may have increased intracellular pools and spared the need to utilize adenosine 5'-triphosphate, which may be limiting due to a lack of a carbon source such as glucose.

Chemical characteristics have already been established for the low-molecular-weight, heat-stable, fluid-accumulating substance produced by ENT+ strains of *E. coli*. Smith and Halls (28) demonstrated the heat stability of an acetone-precipitable, fluid-accumulating material, whereas Jacks and Wu (10) have shown the presence of protein and carbohydrate in a toxin preparation. Furthermore, most of the toxin can be found in the 1,000- to 10,000-molecular-weight range (1, 10), with some activity detected in higher-molecular-weight fractions. The defined medium described in this report should facilitate purification attempts and subsequent studies on the mode of action of ST. The properties of ST produced in the defined medium may be different from those determined in the presence of contaminating macromolecules present in complex media and contaminating cellular constituents due to cell lysis during extended incubation times used by previous investigators (7, 8, 10, 11).

Evidence exists for the synthesis of other heat-stable, low-molecular-weight toxins by *Klebsiella pneumoniae*, *Enterobacter cloacae*, and *Salmonella typhimurium* (12, 13, 24). However, very limited information is available on the nutritional requirements of these and other pathogens (17) for growth and/or toxin synthesis. It would be interesting to know whether synthesis of other similar enterotoxins is inhibited by addition of glucose. Sandefur and Peterson (24) did not detect any synthesis by *S. typhimurium* of heat-stable, rapid-acting skin permeability factor when glucose was used to supplement various complex media. Glucose does not repress synthesis of LT; however, the amino acids required in the presence of glucose are quite different from those required for ST synthesis (P. H. Gilligan and D. C. Robertson, unpublished data).

It is interesting that vigorous aeration does not decrease levels of ST produced by porcine and bovine strains of ENT+ *E. coli*, in contrast to human strains (8) and other enterics that produce ST (12, 13). The mechanism by which aeration shuts off ST synthesis is unknown, but it seems to be coupled to physiology and energy metabolism, as is the repression by glucose.

A better understanding of the host-parasite relationships between humans and neonatal animals and diarrheagenic strains of *E. coli* will be possible once factors controlling enterotoxin synthesis are appreciated.

ACKNOWLEDGMENTS

This work was supported by Public Health Service research grant no. AI-12357 under the U.S.-Japan Cooperative Medical Science Program, administered by the National Institute of Allergy and Infectious Diseases. John Alderete is the recipient of a Ford Foundation Fellowship.

LITERATURE CITED

1. **Bywater, T. J.** 1972. Dialysis and ultrafiltration of a heat-stable enterotoxin from *Escherichia coli*. J. Med. Microbiol. 5:337-342.
2. **Callahan, L. T., III, and S. H. Richardson.** 1973. Biochemistry of *Vibrio cholerae* virulence. III. Nutritional requirements for toxin production and the effects of pH on toxin elaboration in chemically defined media. Infect. Immun. 7:567-572.
3. **Callahan, L. T., III, R. C. Ryder, and S. H. Richardson.** 1971. Biochemistry of *Vibrio cholerae* virulence. II. Skin permeability factor/cholera enterotoxin production in a chemically defined medium. Infect. Immun. 4:611-618.
4. **De Crombrugghe, B., R. L. Perlman, H. E. Varmus, and I. Pastan.** 1969. Regulation of inducible enzyme synthesis in *Escherichia coli* by cyclic adenosine 3', 5'-monophosphate. J. Biol. Chem. 244:5828-5835.
5. **Dorner, F.** 1975. *Escherichia coli* enterotoxin. Purification and partial characterization. J. Biol. Chem. 250:8712-8719.
6. **Duncan, J. L., and G. J. Cho.** 1972. Production of staphylococcal alpha toxin. II. Glucose repression of toxin formation. Infect. Immun. 6:689-694.
7. **Evans, F. G., D. J. Evans, and S. L. Gorbach.** 1973. Identification of enterotoxigenic *Escherichia coli* and serum antitoxin activity by vascular permeability factor assay. Infect. Immun. 8:731-735.
8. **Giannella, R. A.** 1976. Suckling mouse model for detection of heat-stable *Escherichia coli* enterotoxin: characteristics of the model. Infect. Immun. 14:95-99.
9. **Gyles, C. L.** 1971. Heat-labile and heat-stable forms of the enterotoxins from *E. coli* strains enteropathogenic for pigs. Ann. N. Y. Acad. Sci. 176:314-322.
10. **Jacks, T. M., and B. W. Wu.** 1974. Biochemical properties of *Escherichia coli* low-molecular-weight, heat-stable enterotoxin. Infect. Immun. 9:342-347.
11. **Jacks, T. M., B. W. Wu, A. C. Braemer, and B. E. Bidlack.** 1973. Properties of the enterotoxic component in *Escherichia coli* enteropathogenic for swine. Infect. Immun. 7:178-189.
12. **Klipstein, F. A., and R. F. Engert.** 1976. Purification and properties of *Klebsiella pneumoniae* heat-stable

enterotoxin. Infect. Immun. 13:373-381.
13. **Klipstein, F. A., and R. G. Engert.** 1976. Partial purification and properties of *Enterobacter cloacae* heat-stable enterotoxin. Infect. Immun. 13:1307-1314.
14. **Leive, L.** 1965. A nonspecific increase in permeability in *Escherichia coli* produced by EDTA. Proc. Natl. Acad. Sci. U.S.A. 53:745-750.
15. **Leive, L.** 1965. Release of lipopolysaccharide by EDTA treatment of *E. coli*. Biochem. Biophys. Res. Commun. 21:290-296.
16. **Mehlman, I. J., M. Fishbein, S. L. Gorbach, A. C. Sanders, E. L. Eide, and J. C. Olson, Jr.** 1976. Pathogenicity of *Escherichia coli* recovered from food. J. Assoc. Off. Agric. Chem. 59:67-80.
17. **Miller, R. D., and D. Y. C. Fung.** 1973. Amino acid requirements for the production of enterotoxin B by *Staphylococcus aureus* S-6 in a chemically defined medium. Appl. Microbiol. 25:800-806.
18. **Neidhardt, F. C., P. L. Bloch, and D. F. Smith.** 1974. Culture medium for enterobacteria. J. Bacteriol. 119:736-747.
19. **Nolan, R. A.** 1971. Amino acids and growth factors in vitamin-free casamino acids. Mycologia 63:1231-1234.
20. **Nolan, R. A., and W. G. Nolan.** 1972. Elemental analysis of vitamin-free Casamino Acids. Appl. Microbiol. 24:290-291.
21. **Nossal, N. G., and L. A. Heppel.** 1966. The release of enzymes by osmotic shock from *Escherichia coli* in exponential phase. J. Biol. Chem. 241:3055-3062.
22. **Pastan, I., and S. Adhya.** 1976. Cyclic adenosine 5'-monophosphate in *Escherichia coli*. Bacteriol. Rev. 40:527-551.
23. **Sack, R. B.** 1975. Human diarrheal disease caused by enterotoxigenic *Escherichia coli*. Annu. Rev. Microbiol. 29:333-351.
24. **Sandefur, P. D., and J. W. Peterson.** 1976. Isolation of skin permeability factors from culture filtrates of *Salmonella typhimurium*. Infect. Immun. 14:671-679.
25. **Schenkein, I., R. F. Green, D. S. Santos, and W. K. Mass.** 1976. Partial purification and characterization of a heat-labile enterotoxin of *Escherichia coli*. Infect. Immun. 13:1710-1720.
26. **Smith, H. W., and C. L. Gyles.** 1970. The relationship between two apparently different enterotoxins produced by enteropathogenic strains of *E. coli* of porcine origin. J. Med. Microbiol. 3:387-401.
27. **Smith, H. W., and C. L. Gyles.** 1970. The effect of cell free fluids prepared from cultures of human and animal enteropathogenic strains of *E. coli* on ligated intestinal segments of rabbits and pigs. J. Med. Microbiol. 3:403-409.
28. **Smith, H. W., and S. Halls.** 1967. Studies on *Escherichia coli* enterotoxin. J. Pathol. Bacteriol. 93:531-543.