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Nutritional Requirements for Synthesis of Heat-Labile Enterotoxin by Enterotoxigenic Strains of *Escherichia coli*

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Optimal growth conditions have been established for production of heat-labile enterotoxin (LT) by both porcine and human strains of enterotoxigenic (ENT^+) *Escherichia coli*. There were no unusual growth factor requirements, and some strains produced fairly high levels of LT in a basal salts medium containing 0.5% glucose if the pH was carefully controlled. Several amino acids markedly stimulated LT synthesis when added to the basal salts-glucose medium. Methionine and lysine were the most stimulatory for both human and porcine strains. Either aspartic acid or glutamic acid further enhanced LT synthesis in the presence of methionine and lysine, with aspartic acid being more stimulatory for porcine strains and glutamic acid more stimulatory for human strains. There were no apparent vitamin requirements and no unusual cations needed for toxin synthesis except that Fe^{3+} was slightly stimulatory for porcine strains. The stimulation by Fe^{3+} was observed only in the presence of the three amino acids, suggesting that the effect was indirect rather than on toxin synthesis. The carbon source also influenced the yield of LT. Glucose supported maximal synthesis, but other carbon sources which exhibit a high degree of catabolite repression also supported high levels of synthesis. Little or no LT was released below pH 7.0; therefore, because the pH drops during growth from 7.5 to 6.8, even in highly buffered media, it was necessary to adjust the pH to 8.0 to effect complete release of cell-associated toxin. The defined medium containing three amino acids reduced the amount of UV-absorbing material in culture supernatants about fivefold and increased LT activity for various strains from two- to fivefold over a complex Casamino Acids-yeast extract medium. Conditions found to be optimal for synthesis of LT were inhibitory for the heat-stable enterotoxin.

Diarrheal disease in humans and neonatal animals due to enterotoxigenic (ENT^+) strains of *Escherichia coli* results from production by the bacteria of at least two extracellular products referred to as enterotoxins. One enterotoxin is a heat-labile antigenic protein (LT) which appears to exist in several molecular forms and associates with either lipopolysaccharide and/or other outer membrane components (20, 25). The mechanism of action of LT appears to be similar to cholera toxin which acts through stimulation of membrane-bound adenylate cyclase in epithelial cells of the small intestine (21, 22) as well as a variety of nonintestinal cells that also have the GM_1 ganglioside receptor on the cell surface (9, 17, 18, 35). The other enterotoxin is a weakly antigenic heat-stable polypeptide (ST) of 5,000 daltons recently purified to homogeneity and chemically characterized (3). The mechanism of action of ST may involve activation of guanylate cyclase (19).

Several laboratories have reported on the purification and characterization of LT, with 23,000, 80,000, 100,000, and 180,000 being reported for the molecular weight, respectively (11, 13, 14, 34). Furthermore, antisera prepared in each laboratory against their purified toxin showed lack of reactivity or at most partial cross-reactivity by the immunodiffusion technique of Ouchterlony (32). The situation is even more complex since LT made by one strain grown in different media had different molecular weights and antigenic specificities (14). By standardization of growth conditions, it should be easier to compare the chemical properties of LT purified in different laboratories. Another factor complicating purification and chemical characterization is that ENT^+ *E. coli* produce a small fraction of the amount of toxin found in culture supernatants of *Vibrio cholerae* (5, 14).

It seemed that a defined medium would simplify purification of enterotoxin from culture supernatants because most polypeptides and UV-absorbing substances contributed by peptones of complex media would be eliminated. Also, factors affecting release and processing of

the extracellular toxin can be more easily identified and studied. In this report we present data which show that the nutritional requirements for LT synthesis by ENT$^+$ *E. coli* are fairly simple, with the amino acids methionine and lysine being most important. Conditions optimal for synthesis of ST (1) produced little or no LT by LT-ST strains of ENT$^+$ *E. coli*.

MATERIALS AND METHODS

Bacterial strains. The *E. coli* strains of porcine origin and H-10407, a human ENT$^+$ strain, were supplied by Harley Moon of the National Animal Disease Center, Ames, Iowa. The other strains of human origin were obtained from Bradley Sack, Baltimore City Hospital, Baltimore, Md. A non-enterotoxigenic *E. coli* strain 011B4 was obtained from Clarence Buller of this department. All strains produced both LT as well as ST except for 263 and 286C_2, a porcine and human strain, respectively, which produce only LT. All cultures were maintained on Trypticase soy agar slants at 4°C, with monthly transfers, or as lyophilized stocks.

Conditions for growth and enterotoxin production. Cultures were grown aerobically on a New Brunswick rotary shaker (300 rpm) at 37°C in 250-ml Erlenmeyer flasks containing 50 ml of medium. Starter cultures were grown in M-9 minimal medium for 8 to 10 h. Each experimental test medium was inoculated with a volume of starter culture which yielded an initial absorbance of 0.05 at 620 nm. The initial pH was 7.5 for porcine strains and 8.0 for human strains, which decreased 0.80 to 0.90 pH unit in the presence of 0.1 M morpholinopropane sulfonate (MOPS). After 8 h of growth, the pH was adjusted to 7.7 to 8.0 to release cell-associated LT. The incubation was continued for 30 min after addition of 5 N NaOH, at which time samples were withdrawn for growth and assay of enterotoxin activity. Bacteria were removed by centrifugation in sterile centrifuge tubes at 12,000 × *g* for 20 min and refrigerated until assay. All experimental samples were assayed on the same day as the cultures were harvested.

Preparation of media. The complex medium of Casamino Acids (CAA) and yeast extract (YE) was prepared as described by Evans et al. (12) and served as a reference complex medium along with Trypticase soy broth and brain heart infusion broth, both of which have been used for LT production in other laboratories (8, 14, 29).

A synthetic medium was prepared with M-9 minimal salts (31) plus 10 mM *N*-tris(hydroxymethyl)-methyl glycine (tricine) (31), trace salts (12), and 0.5% D-glucose by making 5-fold, 100-fold, and 20-fold stock solutions, respectively. All stock solutions of L-amino acids were 10 mg/ml, except for cystine (2 mg/ml), and used at final concentrations of 200 μg/ml, except for phenylalanine and valine (150 μg/ml); isoleucine (100 μg/ml); and tyrosine, tryptophan, and cystine (50 μg/ml). The vitamin supplement used in preliminary experiments was a 100-fold-concentrated solution sterilized by filtration (Millipore, 0.45 μm), with the final concentrations as follows: pantothenate (0.2 μg/ml), *para*-aminobenzoic acid (0.1 μg/ml), thiamine (1.0 μg/ml), riboflavin (0.25 μg/ml), biotin (0.001 μg/ml), folic acid (0.01 μg/ml), pyridoxine (1.0 μg/ml), and niacin (0.4 μg/ml). The concentrated salts solution was adjusted to pH 7.5 in experiments with porcine strains of ENT$^+$ *E. coli* and pH 8.0 for use with human strains. The 10-fold-concentrated solution of MOPS (1.0 M) was adjusted to pH 7.5 or 8.0 and sterilized by filtration (Millipore, 0.45 μm). The salts were sterilized by autoclaving, followed by aseptic addition of vitamins, MOPS, and glucose.

Toxin assays. The LT activity was determined by the Y-1 murine adrenal tumor assay essentially as described by Donta and Smith (10). Gentamicin (50 μg/ml) was incorporated into the Y-1 culture medium, and all assays were performed in 24-well tissue culture cluster dishes. Each well contained 1 ml of medium and was seeded with 10^5 cells per well. After 2 days of growth, the medium was changed before addition of 0.05 ml of culture supernatant. Steroids were determined fluorometrically (23) by using a standard curve prepared with corticosterone. The percent rounding was observed 4 to 18 h after toxin addition, and steroids were extracted after 18 h of incubation. The monolayers were washed with phosphate-buffered saline and digested with 1 N NaOH for protein determination via the Lowry procedure (26). All results were expressed as nanomoles of steroid per milligram of protein. The Chinese hamster ovary cell assay of Guerrant et al. (17) was employed in preliminary experiments; however, it was not acceptable for screening large numbers of media combinations and detecting small differences in toxin production.

PEL assay. The pigeon erythrocyte lysate (PEL) assay proved to be a more quantitative assay as well as being useful for screening large numbers of media combinations. The procedures for withdrawing blood, removal of leukocytes, and composition of solution A were as described by Gill and King (15). The cell lysate was prepared by freezing the erythrocyte suspension in acetone–solid CO_2 for 90 s with rapid thawing in a 37°C water bath. The dose-dependent activation of adenylate cyclase was performed with 40 μl of lysate; 40 μl of activation cocktail containing ATP (12.5 mM), nictotinamide adenine dinucleotide (12.5 mM), NaCl (0.2 M), and HEPES (*N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid, pH 7.3, 50 mM); and 20 μl of LT previously diluted in 0.15% sodium dodecyl sulfate–0.02 M Na_2HPO_4, pH 7.0. After 30 min of incubation at 37°C, the activation stage of the assay was stopped by addition of 1 ml of ice-cold solution A. The activated membranes were collected by centrifugation at 2,000 rpm for 10 min and resuspended in 50 μl of solution B_1 and 25 μl of solution B_2. Solution B_1 contained NaCl, 95 mM; $MgCl_2$, 24 mM; ethylenediaminetetraacetic acid, 6 mM; dithiothreitol, 0.4 mM; 1-methyl-3-isobutyl xanthine, 0.2 mM; bovine serum albumin, 0.5 mg/ml; and tris(hydroxymethyl)-aminomethane-chloride (pH 8.0), 100 mM. Solution B_2 contained ATP, 8 mM; phosphoenolpyruvate, 40 mM; tris(hydroxymethyl)aminomethane (pH 8.0), 40 mM; pyruvate kinase, 160 μg/ml; and myokinase, 160 μg/ml. After 30 min of incubation at 37°C, the accumulation of cyclic AMP (cAMP) was terminated with 80 μl of 0.25 M sodium acetate buffer, pH 4.0, and 220 μl of distilled water. The samples were frozen until assay of cAMP.

Binding assay for cAMP. All experimental samples were centrifuged at 2,000 rpm for 10 min to remove particulate matter, with a 20-μl sample withdrawn for determination of cAMP. The binding assay of Gilman (16) was used to determine the cAMP content in experimental samples. The binding protein was isolated from beef heart (16) with storage at 10 mg/ml and −80°C. Just before use, a portion of the binding protein was thawed and diluted to the desired concentration with 0.02 M K_2HPO_4, pH 7.0. Calf thymus histone (2 mg/ml) was substituted for protein kinase inhibitor (4). The incubations were stopped by adding 0.5 ml of Dowex-1X8 (200 to 400 mesh) (27). After the Dowex-1X8 had settled, 250 μl was transferred to 4 ml of 3a70B counting solution, and the level of radioactivity was determined with a Packard 3375 liquid scintillation spectrophotometer.

RESULTS

Properties of ENT+ strains selected for nutrition experiments. All strains used in this study were screened before use with both the Y-1 adrenal cell assay and Chinese hamster ovary cell assay, both of which are specific for LT (10, 17), and the suckling mouse assay specific for ST (8). Most of the strains produced both LT and ST, except for 263 and $286C_2$, a porcine and human strain, respectively. Samples of culture supernatants were heated at 65°C for 30 min to verify the presence of a heat-labile molecule active in the assays described above.

LT synthesis in complex and synthetic media. Several complex media, such as brain heart infusion broth, Trypticase soy broth, CAA-YE, and CAA, were examined for their potential to support synthesis of LT and to establish a base line for comparison with combinations of possible required nutrients. Brain heart infusion broth and CAA-YE medium supported higher levels of LT synthesis than did either Trypticase soy broth or CAA medium. However, because brain heart infusion was four- to fivefold higher in protein levels as measured by the Lowry et al. method (26) and CAA-YE has been used in numerous other laboratories, we chose the latter as a standard with which to compare various combinations of synthetic media.

Several tissue culture media, nutrient mixture F-10, RPMI medium 1640, medium 199 (with Earle salts), minimum essential medium (with Earle salts), were examined for their potential to support LT synthesis to determine whether a completely unknown growth factor might be required. Reasonably good growth and LT levels were observed with F-10 and RPMI media supplemented with 50 mM phosphate and 0.3% glucose. If the amino acid components of the tissue culture media were increased to 200 μg/ml, LT synthesis was comparable to CAA-YE medium (data not shown).

Based on our experiments with different tissue culture media, we constructed a complex defined medium containing the 18 amino acids in CAA, 8 vitamins present in YE, salts, 0.5% glucose, and a buffer for pH control, MOPS. Additional buffer capacity was necessary because ENT+ strains of *E. coli* decrease the pH during growth from 7.5 to 5.5 in the presence of 66 mM phosphate. Addition of MOPS yielded a final pH of 6.8 to 6.9 after 8 h of growth. The data in Table 1 show the increase over basal salts due to the amino acid and vitamin components. The response in CAA-YE medium is included for comparison. In general, porcine strains of ENT+ *E. coli* produce less LT in the complex medium than do human strains. It should be noted that the reasonable high toxin levels in the basal salts medium were due primarily to pH control afforded by MOPS. Growth rates were similar in CAA-YE and the defined medium containing amino acids and vitamins.

Correlation of assays specific for LT. The Y-1 adrenal cell assay proved to be too laborious and time consuming to scan large numbers of samples generated by multiple combinations of media components. However, we felt it necessary to verify that changes observed in one assay would be detected in the other because it was possible that only the portion of LT with enzymatic activity, like the A fragment of cholera toxin, would be measured in the PEL assay.

The data in Table 1 show the results of simultaneous assays on undiluted culture supernatants and are expressed as the average of at least three experiments with a variation of ±5%. There was good correlation between the two assays. The PEL assay was the more sensitive of the two, because only 2 μl of culture supernatant was required to produce significant stimulation of adenylate cyclase. The background has been subtracted in this and all subsequent experiments and ranged from 1.2 to 2.0 nmol of steroid per mg of Y-1 adrenal cell protein and 15 to 30 pmol of cAMP in the PEL assay.

Amino acid requirements for LT synthesis. Amino acid requirements were identified by placing them in groups of four as described by Callahan and Richardson (5). A single amino acid was present in two groups, except for proline and lysine, which made it possible to identify stimulatory and inhibitory combinations as well as those with no effect.

There was little or no correlation between growth and levels of LT for both human and porcine strains. The data presented in Table 2 with strain 1362, a porcine strain, are representative of all eight strains examined in this study. Initially, the requirements seemed complex, with numerous antagonistic and synergistic combi-

nations. Groups 4 and 5 were usually the most stimulatory, with groups 1 and 2 and either 7 or 8 giving intermediate degrees of stimulation. Groups 3, 6, and 9 were never stimulatory to porcine strains; however, group 9 was often stimulatory to human strains. As these studies progressed, it became evident that branched-chain amino acids were inhibitory to LT synthesis; thus, proline and lysine were incorporated into new groups. Our approach to identifying those stimulatory amino acids will be outlined with a porcine strain, 1362.

The amino acids common to the stimulatory groups (methionine, histidine, glutamic acid, aspartic acid, threonine, arginine, serine, tyrosine, and glycine) were added to the basal salts medium so that each new group contained one additional amino acid up to a maximum of five (experiments 1, 2, and 3, Table 3). Only the data for the groups containing the maximal number of amino acids are shown to facilitate presentation. As amino acids from the first three groups in Table 3 were eliminated, they were assayed in yet a third combination to confirm that they had no effect on synthesis of LT (experiments 5, 6, and 7). The remaining five amino acids were then examined in detail. Although histidine showed an additive effect in the presence of methionine and lysine, it did not increase the maximal effect observed with methionine, lysine, and either aspartic acid or glutamic acid. The data obtained with representative porcine and human strains, by the approach just described for strain 1362, are summarized in Tables 4 and 5, respectively. Some amino acids showed a small degree of stimulation in the absence of either methionine or lysine; for example, glycine, serine, and arginine, in addition to histidine, showed a moderate enhancement for porcine strains, whereas cystine and proline had a similar effect on human strains. However, none had an additional effect in the presence of methionine, lysine, and either aspartic acid or glutamic acid.

The basis for the antagonism between certain amino acids as just described is unknown, but was consistently observed throughout the course of this work. The most dramatic stimulation was seen with methionine and lysine, with either aspartic acid or glutamic acid providing maximal LT levels. Addition of any of the other amino acids which appeared stimulatory in the absence of either methionine or lysine singly or in combination proved to be somewhat inhibitory. The levels of LT produced by growth in the presence of the three amino acids just noted was consistently higher than the initial mixture of 18 amino acids, which again points to the inhibitory nature of certain amino acids. Once the amino acid requirements were established, there appeared to be no vitamin requirement (data not shown).

Effect of pH on LT synthesis. The effect of pH on LT release into the supernatant was of concern because Dorner reported that the enterotoxin was rapidly inactivated below pH 6.9 (11). Even with addition of 0.1 M MOPS, the pH dropped to 6.7 to 6.8 for porcine strains and 7.1 to 7.2 for human strains. It should be noted that the initial pH was 8.0 and 7.5 for human and porcine strains, respectively. Other studies on the mechanism of release of LT from ENT$^+$ *E. coli* have indicated that minimal LT is released below pH 7.3 in a defined medium containing three amino acids (S. L. Kunkel and D. C. Robertson, unpublished data). It made no difference whether the pH was maintained at 7.5 to 7.7 throughout growth or whether it was adjusted

TABLE 1. *LT synthesis by ENT$^+$ E. coli strain 1362 in various media*

Expt	Medium	OD_{620}[a]	pH	LT level	
				Steroid (nmol/mg of protein)[b]	cAMP[c] (pmol)
1	Salts + 0.5% glucose	3.2	6.95	8.2	128
2	As in expt 1 + 18 amino acids + 8 vitamins	5.1	6.8	22.5	220
3	CAA-YE	5.1	8.6	9.7	110

[a] OD_{620}, Optical density at 620 nm.
[b] A 50-μl amount of culture supernatant added to each well.
[c] Equivalent of 2 μl of culture supernatant used in each assay.

TABLE 2. *Effect of amino acid mixtures on LT synthesis by ENT$^+$ E. coli strain 1362*

Mixture no.	OD_{620}[a]	Toxin activity[b]	Content
1	4.4	9.5	His, Phe, Glu, Arg
2	4.4	9.5	Gly, Leu, Tyr, Ser
3	4.5	6.4	Cys, Ile, Trp, Ala
4	3.9	14.0	Met, Val, Thr, Asp
5	5.0	15.5	His, Gly, Cys, Met
6	4.6	6.8	Phe, Leu, Ile, Val
7	4.8	11.0	Glu, Tyr, Trp, Thr
8	4.6	9.5	Arg, Ser, Ala, Asp
9	4.2	5.7	Pro, Lys, Ala, Ile
10	3.8	7.9	Salts
11	6.1	20.0	Salts, 18 amino acids

[a] OD_{620}, Optical density at 620 nm.
[b] Nanomoles of steroid per milligram of Y-1 adrenal protein.

to 7.7 to 8.0 in one step after 8 h of growth. Because the latter was more convenient, all experiments in the later stages of this work were carried out with a single pH adjustment. The data in Table 6 show the effect of pH adjustment on LT synthesis by a human strain, H-10407. It is of interest that significant amounts of LT were released from human strains with the medium containing 18 amino acids in the pH range of 7.1 to 7.3. Perhaps there are nutritional factors that stimulate the release of LT.

Effect of cations on LT synthesis. The contribution of cations to LT synthesis was tested because these salts were present in both CAA-YE medium and the synthetic medium containing 18 amino acids. There was no growth in the absence of Mg^{2+}; thus, all experiments examined the effect of Mn^{2+} and Fe^{3+} in the presence of Mg^{2+}. The data in Table 7 show that Mn^{2+} was inhibitory to LT synthesis for porcine strains of ENT+ *E. coli*, whereas Fe^{2+} was stimulatory. The stimulation was likely metabolic in nature because there was no effect in a salts medium, only in the presence of amino acids. Ferric ion had no effect on LT synthesis by human strains, but slightly increased growth. The micronutrient mixture of Neidhardt et al. (31) had no effect on LT synthesis. The concentrations of Fe^{2+} found to be optimal for porcine strains was the same as in CAA-YE medium (12).

TABLE 3. *Amino acid requirements of ENT+ E. coli strain 1362*

Expt	Additions	cAMP (pmol)
1	Salts	86
2	Salts + Met + His + Glu + Asp + Thr	217
3	Salts + Arg + Ser + Tyr + Gly	87
4	Salts + Pro + Lys + Ala	143
5	Salts + Pro + Ala + Thr + Try + Cys	86
6	Salts + Arg + Ser + Try + Gly + Phe	90
7	Salts + Leu + Ile + Val	102
8	All 18 amino acids	170
9	Salts + Met	125
10	Salts + Met + His	178
11	Salts + Met + Lys	190
12	Salts + Met + Lys + His	237
13	Salts + Met + Lys + Asp	280
14	Salts + Met + Lys + Glu	240
15	Salts + Met + Lys + Glu + His	170
16	Salts + Met + Lys + Glu + Asp	310

Carbon sources and LT synthesis. Because the synthesis of ST has been shown to be subject to catabolite repression (2), several carbon sources were examined for their effect on LT production (Table 8). The results were just opposite to those observed in nutrition studies on ST; that is, those sugars which exhibited the highest levels of catabolite repression supported the highest levels of LT production. The role(s) of glucose in LT synthesis was further examined by adding glucose to a mixture of amino acids optimal for ST production and a mixture containing ST amino acids and amino acids which stimulated LT synthesis. There was no appreciable LT detected until glucose was added, which would have the effect of inhibiting ST synthesis (2). It was not feasible to increase the glucose concentration above 0.5% due to the acid production and drop in pH, even in the presence of MOPS.

Kinetics of LT synthesis and release. The release of LT during growth was followed to determine the rate of release and ascertain at what point maximal synthesis occurred (Fig. 1). There was a good correlation between growth and toxin production in the CAA-YE medium where the pH did not drop below 7.8. In the defined medium containing three amino acids, the bulk of the toxin was detected in the late-log phase when the pH was maintained above 7.5. Somewhat higher LT levels were consistently

TABLE 4. *Amino acid requirements for LT synthesis by porcine strains of ENT+ E. coli*

Addition	OD_{620}[a] for strain:				cAMP (pmol) for strain:			
	263	1288	1291	1362	263	1288	1291	1392
Salts	4.4	3.0	3.2	3.4	68	166	97	86
+ Met	4.8	3.8	4.1	3.8	103	218	146	125
+ Met + Lys	4.4	3.4	3.9	3.2	188	194	205	190
+ Met + Lys + Asp	4.6	3.5	3.4	3.2	265	178	200	280
+ Met + Lys + Glu	4.5	ND[b]	3.6	3.4	230	ND	225	240
+ Met + Lys + Glu + Asp	4.8	ND	3.6	3.6	ND	ND	265	310
+ 18 amino acids	5.0	4.4	5.3	5.1	133	214	149	170
CAA-YE	4.2	4.8	5.7	4.3	56	62	83	66

[a] OD_{620}, Optical density at 620 nm.
[b] ND, Not done.

TABLE 5. *Amino acid requirements for LT synthesis by human strains of ENT⁺ E. coli*

Addition	OD_{620} for strain:				cAMP (pmol) for strain:			
	$286C_2$	334	408-3	H-10407	$286C_2$[b]	334	408-3	H-10407
Salts	2.7	3.3	3.5	2.5	77	59	54	96
+ Met	3.3	3.8	4.1	2.8	93	89	57	105
+ Met + Lys	2.0	3.8	3.6	3.0	185	197	205	134
+ Met + Lys + Asp	2.9	3.8	ND[c]	3.6	212	279	ND	187
+ Met + Lys + Glu	2.9	3.9	3.8	3.6	259	193	264	257
+ 18 amino acids	5.2	5.5	3.8	4.9	112	244	187	210
CAA-YE	4.75	5.4	6.8	5.2	74	89	177	145

[a] OD_{620}, Optical density at 620 nm.
[b] Diluted 1:16 in salts medium before dilution in 0.02 M PO_4^{3-}–0.15% sodium dodecyl sulfate, pH 7.0.
[c] ND, Not done.

TABLE 6. *Effect of pH on LT synthesis by ENT⁺ E. coli strain H-10407*

Expt	OD_{620}[a]	pH	LT Levels	
			Steroid nmol/mg of protein	cAMP (pmol)
1. Basal salts	3.5	7.2	2.6	49
2. As 1—pH adjustment		8.35	2.8	67
3. As 1 + Met + Lys + Gln	4.35	6.3	3.9	10
4. As 3—pH adjustment		8.0	14.7	289
5. As 1 + 18 amino acids	5.5	7.1	9.2	143
6. As 5—pH adjustment		7.9	9.3	144
7. CAA-YE	5.4	8.5	8.9	181

[a] OD_{620}, Optical density at 620 nm.

observed with a single pH adjustment compared with adjustment when necessary to keep the pH above 7.5. It may be that some inactivation occurs due to proteolytic activity or association with lipopolysaccharide. We always found maximal LT levels at 8 to 9 h of growth; therefore, we can see no advantage to longer incubation times which will also promote cell lysis.

DISCUSSION

Nutritional conditions for maximal synthesis of LT by human and porcine strains of ENT⁺ *E. coli* have been established. Because *E. coli* has no known growth factor requirements, the role of amino acids, and Fe^{3+} in the case of porcine strains, was to increase basal levels synthesized in M-9 minimal medium. The pH and carbon source influence the release of LT, as do several other parameters (Kunkel and Robertson, unpublished data). If the pH was controlled in M-9 minimal medium by addition of 0.1 M MOPS, LT levels for six of eight strains described in this

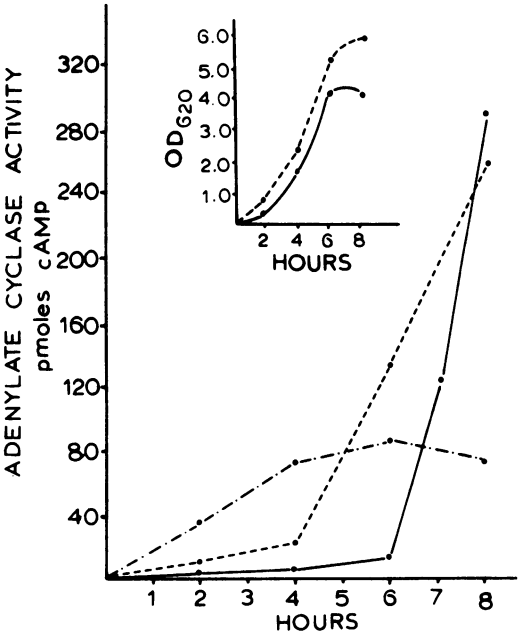

FIG. 1. *Rate of LT synthesis by ENT⁺ E. coli strain 1362 grown in various media. Symbols: LT activity in CAA-YE medium (·····); LT activity in M-9 salts + 0.5% glucose + 0.1 M MOPS + three amino acids (methionine, lysine, and aspartic acid) with pH adjusted during growth (---); and LT activity in M-9 salts + 0.5% glucose + 0.1 M MOPS + three amino acids (methionine, lysine, and aspartic acid) with pH adjusted at 7.5 h (——). Insert shows growth (OD_{620} [optical density at 620 nm]) in CAA-YE medium (---) and M-9 salts + 0.5% glucose + 0.1 M MOPS + three amino acids (methionine, lysine, and aspartic acid) with pH adjusted at 7.5 h (——).*

report were similar to a complex medium containing CAA and YE. The use of defined reference strains and standard growth conditions should make it easier to compare LT preparations and antisera in various laboratories.

Two amino acids, methionine and lysine, markedly stimulated synthesis of LT, with either

TABLE 7. *Effect of divalent cations on LT synthesis by porcine strains of ENT+ E. coli*

Ion content	OD_{620} for strain:				cAMP (pmol) for strain:			
	263	1288	1291	1362	263	1288	1291	1362
Mg^{2+}	3.6	2.8	3.5	2.8	184	154	138	93
$Mg^{2+} + Fe^{3+}$	3.4	3.3	3.6	3.2	254	234	270	155
$Mg^{2+} + Mn^{2+}$	3.6	3.3	3.7	3.3	106	184	170	67
$Mg^{2+} + Fe^{3+} + Mn^{2+}$	3.6	3.4	3.7	3.5	236	118	190	159

TABLE 8. *Effect of carbon source on LT production by ENT+ E. coli strain 1362*

Expt	Carbon source	OD_{620}[a]	pH	cAMP (pmol)
1	Lactose	3.8	6.7	131
2	Sucrose	3.4	7.1	129
3	Glucose	3.6	6.7	200
4	Gluconate	2.8	7.1	210
5	Galactose	4.6	6.75	140
6	L-Arabinose	3.5	6.75	180
7	Glycerol	4.4	6.8	73
8	Pyruvate	2.6	7.3	69
9	ST amino acids[b]	3.3	7.8	20
10	ST + LT amino acids[c]	3.5	8.0	22
11	As 10 + 0.5% glucose	4.8	6.7	190

[a] OD_{620}, Optical density at 620 nm.

[b] Contained L-proline, 12.4 mM; L-aspartic acid, 6.6 mM; L-alanine, 4.4 mM; and L-serine, 6.2 mM in addition to 10 mM tricine and M-9 salts.

[c] Methionine, 200 μg/ml; lysine, 200 μg/ml; and aspartic acid, 200 μg/ml.

glutamic acid or aspartic acid supporting the highest levels. Aspartic acid appeared to be more stimulatory than glutamic acid for porcine strains, with just the opposite for human strains; however, because the two amino acids were slightly synergistic, both can be included to meet the broadest range of circumstances. The branched-chain amino acids (leucine, isoleucine, and valine) were often inhibitory, as was histidine for some strains. In all our nutritional work, eight amino acids were most often stimulatory in various combinations; thus, any nutrition experiment on recent isolates could start with methionine, lysine, aspartic acid, glutamic acid, arginine, serine, proline, and histidine. Synthesis of LT was always higher in the presence of three amino acids, instead of the 18 present in CAA, and several-fold higher than in CAA-YE medium. These data show the inhibitory nature of some amino acids. A porcine strain, 1288, produced near maximal levels of LT in M-9 medium and was stimulated by only methionine. Two human strains, 408-3 and H-10407, produced fairly high toxin levels in CAA-YE medium in contrast to the other six strains. Once the stimulatory amino acids were identified, it was not possible to demonstrate any requirement for vitamins.

The role(s) of Fe^{3+} in LT synthesis was examined because of the importance of the cation in microbial infections and its effect in synthesis of bacterial exotoxins, for example, cholera toxin (6, 33) and diphtheria toxin (7). It had no effect on LT synthesis by human strains, but was somewhat stimulatory for porcine strains. The effect was likely metabolic because the stimulation could be demonstrated only in the presence of the necessary amino acids. Inhibition by Mn^{2+} was observed with porcine strains and had no effect on human strains; thus, it was omitted from most nutrition experiments. The metal chelator tricine was employed to solubilize both Fe^{3+} and Mn^{2+} (33).

All strains of ENT+ *E. coli* examined in our laboratory produce large amounts of acid when grown in M-9 or a defined medium containing 0.5% glucose and drop the pH to as low as 5.5 in the presence of 66 mM phosphate. The low levels of LT detected under these conditions might be explained by inactivation of the toxin. Addition of 0.1 M MOPS prevented the pH from dropping below 6.8 when the initial pH was 7.5. However, early in these studies, it was observed that release of LT was inhibited below pH 7.3. The pH of most nutrition experiments was adjusted to 7.7 to 8.0 after 8 h of growth with an additional 30-min incubation at 37°C for complete release of cell-associated LT. Although not examined in detail, LT release in the presence of the 18-amino acid mixture was greater in the pH range of 7.1 to 7.3. Some amino acids may facilitate release of LT through induction of a protease, as has been observed in other systems (28). The critical pH for release of LT by ENT+ *E. coli* is about 0.5 unit lower than for the release of cholera toxin by *V. cholerae*. These data help to explain the observations of Mundell et al. (30) in which it was observed that LT levels after growth were a function of the initial pH. The pH drops and then rises in a complex medium; therefore, the less time it is below 7.3 to 7.5, the greater the release of LT. The basis of the pH effect and its role in the release of LT are not known at the present time. Callahan and Rich-

ardson (5) suggested that ionic interactions were important in the release of cholera toxin by *V. cholerae*. An alternative hypothesis is that pH adjustment is necessary to permit a protease to complete the transport of the toxin to the outer surface of the bacterium, as has been seen in the release of penicillinase from *B. licheniformis* (24) and alkaline phosphatase from *E. coli* (28). In fact, protease inhibitors will partially block LT release if added before pH adjustment (Kunkel and Robertson, unpublished data).

Because pH control was a problem with glucose as the carbon source, other carbohydrates were examined for the potential to support LT synthesis. Those sugars which exhibit catabolite repression by lowering cAMP levels supported the highest levels of toxin synthesis. It is interesting that conditions which turn on synthesis of LT are inhibitory to ST synthesis (2). The nutritional conditions for maximal synthesis of LT are quite different than those necessary for synthesis of ST (2) in which it was shown to be subject to glucose repression, whereas glucose is required for LT synthesis and release. Addition of those amino acids which stimulated LT synthesis to the four amino acids required for ST synthesis had no effect until glucose was added to the amino acid mixture.

Temperature has also been shown to exert an effect on the structure and/or processing of LT (Kunkel and Robertson, unpublished data). When grown at 22°C, ENT$^+$ *E. coli* did not release cell-associated LT upon pH adjustment; however, polymyxin B extraction yielded a fragment of 30,000 daltons with biological activity only in the PEL assay. The release of cell-associated LT increased gradually starting at 26°C and reached a maximum at 37°C. The LT released by pH adjustment had biological activity in both the PEL and Y-1 adrenal tumor cell assays and appeared to be the holotoxin form of LT with a molecular weight of 73,000. Finally, vigorous aeration supported optimal synthesis of LT (data not shown).

In our opinion, the nutritional parameters defined in this work will complement attempts to produce hypertoxigenic ENT$^+$ mutants. There seems to be little question that the low yield of extracellular toxin has been a contributing factor to conflicting molecular weights and heterogeneity of purified preparations (11, 13, 14, 29, 34). One human strain, 286C$_2$, produces at least 30-fold more extracellular toxin than does H-10407, a strain used to study various aspects of the pathogenesis of ENT$^+$ *E. coli* (12). Most important, the amount of stimulation afforded by the amino acids methionine, lysine, and glutamic acid, compared with the basal salts-glucose medium, was similar to other strains which produced low levels of LT. Experiments are in progress to determine the reasons for the high amounts of LT produced by strain 286C$_2$. Using the defined medium just described, we have been able to purify to homogeneity extracellular LT produced by both human and porcine strains of ENT$^+$ *E. coli* (Kunkel and Robertson, manuscript in preparation). The defined medium can be used for production of LT under fermentor conditions and vigorous aeration without the use of MOPS because the pH does not drop below 6.8. It appears that it is possible to increase the homogeneity of crude and purified LT preparations and decrease the bewildering array of multiple-molecular-weight forms and apparently different antigenic species.

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