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J. Bacteriol. **1974, 119(3):736.**

JOURNAL OF BACTERIOLOGY, Sept. 1974, p. 736-747

Vol. 119, No. 3
Printed in U.S.A.

Culture Medium for Enterobacteria

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Received for publication 29 April 1974

A new minimal medium for enterobacteria has been developed. It supports growth of *Escherichia coli* and *Salmonella typhimurium* at rates comparable to those of any of the traditional media that have high phosphate concentrations, but each of the macronutrients (phosphate, sulfate, and nitrogen) is present at a sufficiently low level to permit isotopic labeling. Buffering capacity is provided by an organic dipolar ion, morpholinopropane sulfonate, which has a desirable pK (7.2) and no apparent inhibitory effect on growth. The medium has been developed with the objectives of (i) providing reproducibility of chemical composition, (ii) meeting the experimentally determined nutritional needs of the cell, (iii) avoiding an unnecessary excess of the major ionic species, (iv) facilitating the adjustment of the levels of individual ionic species, both for isotopic labeling and for nutritional studies, (v) supplying a complete array of micronutrients, (vi) setting a particular ion as the crop-limiting factor when the carbon and energy source is in excess, and (vii) providing maximal convenience in the manufacture and storage of the medium.

Escherichia coli and *Salmonella typhimurium* are probably the most completely studied organisms in the world, and recent genetic and physiological work has encouraged the thought that these organisms may be essentially solved within a couple of decades.

Several obstacles stand in the way of this goal. One is the disturbing fact that only some of the qualitative information about *E. coli* or *S. typhimurium* cells, and virtually none of the quantitative information, can yet be integrated into whole-cell models. Physiologists have failed to settle upon a single strain to study, and they have used a great variety of growth media and conditions. Different strains have different growth rates, and the lack of an agreed-upon recipe for a minimal medium has compounded the difficulty. Furthermore, stories are legion of investigators finding that the growth rate of their strain has inexplicably changed, sometimes upon moving to another laboratory, sometimes with no identifiable concomitant event.

To overcome this difficulty attention must be directed to three aspects of the culture—the cell strain, the medium, and the several techniques and procedures used in the cultivation. We are currently developing strains of *E. coli* K-12, *E. coli* B, and *S. typhimurium* LT2 which we hope soon to nominate as standard strains. Here we present a new medium, together with a few rules and conventions for its use, which offers sufficient advantage over currently used media to justify universal acceptance by physiologists studying the enterobacteria.

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MATERIALS AND METHODS

Organisms. Several strains of *E. coli* B, B/r, K-12, and *S. typhimurium* LT2 were used to test the various formulations of the minimal medium because it was deemed both possible and desirable to construct a single medium for both of these enterobacterial species. For the *E. coli* B and B/r strains, several substrains were used; for clarity and simplicity these are not separately identified. The particular substrain of *S. typhimurium* LT2 used, however, has been given a new designation, NT1. Strains of LT2 obtained from different laboratories were found to have different growth properties; strain NT1 is prototrophic and was selected for rapid growth and smooth dispersion of cell cultures.

Media. Various minimal media were used for comparative purposes, and these are described in Table 1, including the final formulation of the new medium. Detailed directions for preparing the new media are given here; supplementary information appears in the Results and Discussion sections.

Preparation and use of MOPS medium. (i) Prepare 1 liter of 10× concentrate by mixing the following solutions in the given order to prevent precipitation of various salts: potassium morpholinopropane sulfonate (MOPS), freshly prepared, 1.0 M, adjusted to pH 7.4 with KOH (400 ml); *N*-Tris(hydroxymethyl)-methyl glycine (Tricine), freshly prepared, 1.0 M,

TABLE 1. *Composition of various media*

Component[a]	Concn in medium (mM)							
	M9[b]	M63[b]	Modified Werkman[c]	Vogel-Bonner[d]	A[b]	Davis-Mingioli[e]	Tris[f]	MOPS
KH_2PO_4	22	100	100		33	22		
K_2HPO_4				73	60	51	0.1	1.32
Na_2HPO_4	42		50					
$NaNH_4HPO_4$				17				
$(NH_4)_2SO_4$		15	15		8	8		
NH_4Cl	19						20	9.52
Na_2SO_4							2.5	
$MgSO_4$	1	1	1	0.8	1	0.4		
$MgCl_2$								0.523
K_2SO_4								0.276
$FeSO_4$[g]		2×10^{-3}						0.010
$FeCl_3$							2×10^{-3}	
Citrate				10	2	2		
$CaCl_2$	0.09		0.09				0.35	5×10^{-4}
NaCl	9						80	50
KCl							20	
MOPS								40
Tris							120	
Tricine								4
Micronutrients[h]								+

[a] For simplicity, components differing only in degree of hydration are combined.
[b] Reference 9.
[c] Reference 4.
[d] Reference 10.
[e] Reference 2.
[f] Reference 7.
[g] The heptahydrate salt is recommended.
[h] $(NH_4)_6(MO_7)_{24}$, 3×10^{-6} mM; H_3BO_3, 4×10^{-4} mM; $CoCl_2$, 3×10^{-5} mM; $CuSO_4$, 10^{-5} mM; $MnCl_2$, 8×10^{-5} mM; $ZnSO_4$, 10^{-5} mM.

adjusted to pH 7.4 with KOH (40 ml); $FeSO_4$, freshly prepared, 0.01 M (10 ml); NH_4Cl, 1.90 M (50 ml); K_2SO_4, 0.276 M (10 ml); $CaCl_2$, 5.0×10^{-4} M (10 ml); $MgCl_2$, 0.528 M (10 ml); NaCl, 5.0 M (100 ml); micronutrients [a solution containing $(NH_4)_6(MO_7)_{24}$, 3×10^{-6} M; H_3BO_3, 4×10^{-4} M; $CoCl_2$, 3×10^{-5} M; $CuSO_4$, 10^{-5} M; $MnCl_2$, 8×10^{-5} M; $ZnSO_4$, 10^{-5} M] (10 ml); and glass-distilled water (360 ml). Total amount is 1,000 ml.

(ii) Filter sterilize (e.g., with Nalgene disposable filter units, 0.20 μm) this solution, using a filter prerinsed with a small amount (10 ml) of the solution. This sterile 10× medium concentrate lacks only a carbon source and phosphate (which would precipitate at this concentration); it may be stored for long periods (at least 2 years) at −20 C.

(iii) For each liter of MOPS medium, aseptically mix: 10× MOPS concentrate (100 ml); K_2HPO_4, 0.132 M (10 ml); carbon source (100×) (10 ml); and glass-distilled water, autoclaved (880 ml). Total amount is 1,000 ml. Alternatively, culture flasks can be conveniently prepared by aseptically adding appropriate volumes of 10× MOPS concentrate and phosphate and carbon source to culture flasks previously autoclaved with the desired amount of water.

(iv) Comments on the preparation: The MOPS, Tricine, and $FeSO_4$ solutions should be freshly prepared for making the concentrate; all other solutions can be stored, but their prolonged storage is not recommended because of the possibility of microbial growth. The micronutrient solution is best prepared by combining appropriate volumes of more concentrated solutions of the individual components. The final pH of the medium will be close to 7.2. The medium can be solidified by aseptically adding appropriate volumes of 10× MOPS concentrate and phosphate and carbon source to sterile molten agar solution of the desired concentration.

Cultivation of cells. Cultures (25- or 50-ml volume) were grown aerobically in Erlenmeyer flasks (125- or 250-ml capacity, respectively) capped with Morton-type metal closures. The shaking water baths (New Brunswick Scientific Co., model G-76) were equipped with modified temperature controls (Yellow Spring Instruments Co., Thermistemp Temperature Controllers, model 71, 71A, or 72) that maintained the temperature at 37 ± 0.3 C. The platform rotation was approximately 240 rpm (setting no. 6).

Measurement of growth. Many of the nutritional studies were conducted by inoculating (usually from a rich-agar slant, but preferably from a frozen culture of cells in MOPS medium) a flask containing medium with sufficient glucose to permit growth only to 1.0 absorbance unit (1-cm light path, 420 nm [A_{420}]). On

the following day this culture was used to inoculate experimental flasks at an absorbance between 0.01 and 0.02 units. Only data obtained after at least three generations of growth and between 0.1 and 1.0 A_{420} unit was used to calculate growth rates; for many purposes longer periods of steady state growth are recommended.

Growth was followed by periodically transferring measured samples (0.5 to 2.0 ml) of culture to a tube containing 0.5 ml of 0.9% (vol/vol) formaldehyde to stop growth. These samples were then diluted with water to obtain A_{420} readings between 0.1 and 0.4 (Zeiss Spectrophotometer, model PMQ II, 1-cm light path). This method gave results identical to those obtained with unfixed cells and permitted the absorbance measurements to be made at the experimenter's convenience. (Absorbance of HCHO-treated suspensions were unchanged up to a day later if stored at 4 C.) Growth rate was expressed as the specific first-order rate constant (k) in dimensions of h^{-1}:

$$k = \frac{\ln 2}{\text{doubling time}}$$

The first-order rate constant, k, was obtained as the least-squares plot of the natural log of A_{420} as a function of time in hours. This value was calculated with the aid of a computer by using a transformation of the following standard equation:

$$k = \sum_i (X_i - \bar{x})(Y_i - \bar{y}) / \sum_i (X_i - \bar{x})^2$$

where X_i is time, Y_i is natural log of A_{420}, $\bar{x}$ is average value for all time values, $\bar{y}$ is average value for all $\ln(A_{420})$, $\bar{x}$ is $1/n \sum X_i$, $\bar{y}$ is $1/n \sum Y_i$, and n is number of points in plot.

Measurement of dry weight. A sample of culture (usually 50-ml volume at an absorbance of 1.0 A_{420} unit) was chilled to below 5 C and centrifuged to pellet the cells. The pellet was resuspended in water and again centrifuged. The sedimented cells were quantitatively transferred to washed, dried, and preweighed membrane filters (Millipore, type HA, 0.45-μm pore). These samples were then weighed after drying in vacuo to constant weight over P_2O_5. The results appear in Table 2.

Total cell count. Samples of culture (usually 1.0-ml volume and an absorbance of 1.0 A_{420} unit) were diluted 100-fold with a prefiltered solution containing 0.9% (wt/vol) NaCl and 0.4% (vol/vol) HCHO. These samples were counted by standard methods with an electronic particle counter (Coulter Electronics Co., model B) (Table 2).

pH measurement. The pH of media and other solutions was measured with a glass electrode pH meter (Radiometer, model 26) at room temperature.

Chemicals. All inorganic chemicals were reagent grade and were obtained from normal commercial sources. MOPS and Tricine were obtained from Sigma Chemical Co. Water was double glass-distilled.

RESULTS

MOPS buffer. Over a broad range of concentration up to 80 mM, MOPS buffer was not inhibitory to the growth of enterobacteria. A titration curve of medium made with this buffer revealed considerable buffering capacity from pH 6.2 to 8.0 (Fig. 1). At a concentration of 40 mM, the aerobic growth of enterobacteria on glucose from an inoculum of a few cells per milliliter could be maintained up to an A_{420} of 2.0 (0.286 mg [dry weight]/ml; 6.6×10^8 cells/ml) with a drop of only 0.2 pH units, and an A_{420} of 4.0 with a drop of only 0.4 pH units (Fig. 2). The bacterial strains of interest were found to exhibit little change in growth rate response to initial pH over the range pH 6.5 to 8.0 (Table 3). From this information we decided to use 40 mM MOPS in the medium and to set the initial pH at 7.2.

To provide sufficient chelating capacity for the $Fe^{2,3+}$ required in the medium (Fig. 3), Tricine was added to a final concentration of 4 mM. This addition did not affect the growth of the cells but did prevent precipitation of iron salts (present in the medium at 10^{-2} mM and in the storage concentration at 10^{-1} mM).

The elemental composition of MOPS buffer dictated that it be tested as a potential source of carbon (and energy), nitrogen, and sulfur. Cul-

TABLE 2. *Macronutrient requirements*

Component	Concn to yield unit cell density[a] (mM/A_{420})	Concn in MOPS medium (mM)	Theoretically possible cell yield	
			Absorbance (A_{420})	Dry weight (mg/ml)
Glucose	1.89 ± 0.05	19.84	10.5 (1.05)	1.50 (0.15)
PO_4	0.126 ± 0.001	1.32	10.5	1.50
SO_4	0.0265 ± 0.0006	0.276	10.5	1.50
Mg	0.0168 ± 0.0004	0.528	31.5	4.50
NH_4	1.36 ± 0.02	9.52	7.0	1.00

[a] A culture of *S. typhimurium* in exponential growth in glucose-MOPS medium (k = 1.0 h^{-1}) at an absorbance (A_{420}) of 1.00 contains (3.28 ± 0.06) × 10^8 cells per ml (the standard error is based on six determinations), and (1.44 ± 0.047) × 10^2 μg (dry weight) per ml (the standard error is based on three determinations).

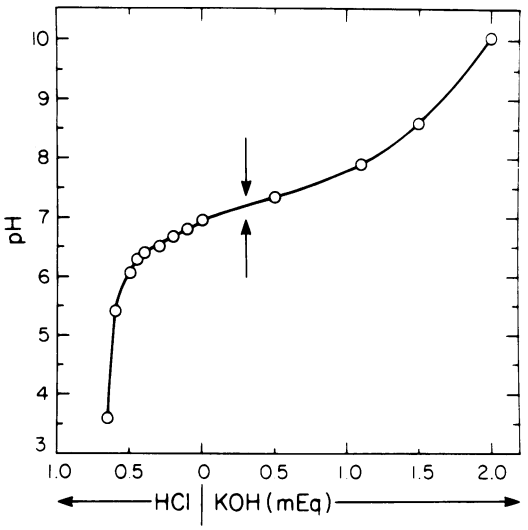

FIG. 1. *Titration of MOPS medium with acid and with base. A solution of 50 ml of MOPS medium was prepared at an initial pH of 7. The pH values resulting from the addition of various amounts of KOH or HCl are plotted as a function of the milliequivalents of acid or alkali added.*

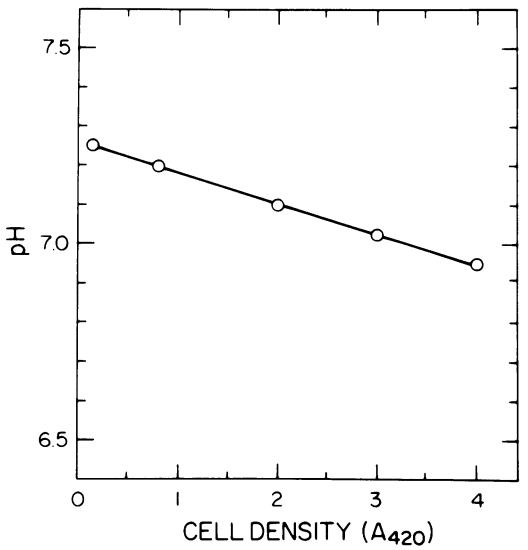

FIG. 2. *Effect of cell growth on the pH of glucose-MOPS medium. A flask containing 50 ml of glucose-MOPS medium at an initial pH of 7.3 was inoculated at a low density (A_{420} = ~0.01) with cells of S. typhimurium NT1. The culture was incubated aerobically at 37 C, and growth was followed spectrophotometrically. Measurements of pH were made, and the results were plotted as a function of cell density. The culture remained in apparent exponential growth to an optical density at 420 nm of approximately 4.0. Above this density growth began to decelerate and the pH rapidly dropped.*

TABLE 3. *Effect of initial pH on growth rate of S. typhimurium NT1*

Initial pH	Growth rate constant, k^a (h^{-1})
6.0	0.82
6.5	0.96
7.0	0.96
7.5	0.94
8.0	0.96

[a] Single flasks at each indicated initial pH were used. In each case the standard deviation of the k values was <0.01 h^{-1}.

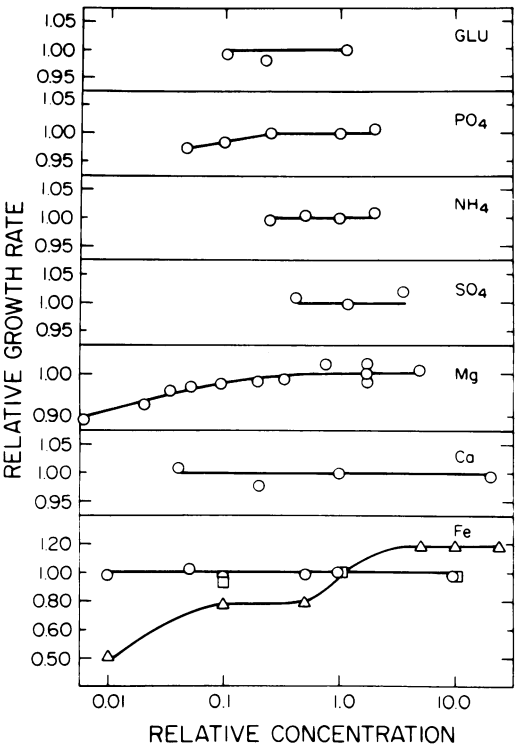

FIG. 3. *Effect of nutrient concentration on the growth rate of S. typhimurium NT1 in glucose-MOPS medium. Growth rates (on the ordinate) are expressed relative to the rate measured in MOPS medium of normal composition. The concentration of each variable nutrient (GLU [glucose], PO_4, NH_4, SO_4 Mg, Ca, Fe) is expressed on logarithmic scale on the abscissa relative to its concentration in normal MOPS medium described in Table 1. For Fe, results are shown with three carbon sources: glucose (□), glycerol (○), and acetate (△).*

tures of *E. coli* B and *S. typhimurium* NT1 were prepared in MOPS medium containing reduced amounts of either glucose, glycerol (in the absence of glucose), NH_4Cl, or K_2SO_4. The

growth of *S. typhimurium* NT1 ceased in each case upon exhaustion of the designated carbon, nitrogen, or sulfur source, and the final crop of cells was proportional to the limiting component. Apparently, MOPS buffer cannot be degraded by these cells into growth-supporting derivatives. In the case of *E. coli* B, the same result was obtained upon limiting the carbon (glucose or glycerol) or nitrogen (NH_4Cl) supply, but cultures growing with limiting amounts of the principal sulfur source (K_2SO_4) exhibited diauxic growth (Fig. 4). Upon exhaustion of the added sulfate, there was a 10-min lag followed by growth at approximately 70% of the normal rate with ample K_2SO_4. MOPS buffer, therefore, can serve as a sulfur source for *E. coli* B.

Experiments were next performed to learn whether *E. coli* B uses MOPS and sulfate simultaneously or sequentially, since the former mode would preclude using $S^{35}[SO_4]$ label with this strain. We used the fact that *S. typhimurium* NT1 cannot use MOPS as a sulfur source. A culture of the coli strain was grown in glucose-MOPS medium lacking K_2SO_4. Samples were taken at a number of turbidities, chilled, and cleared of bacteria by centrifugation and filtration. The sterile medium was then tested for its ability to support growth of *S. typhimurium* NT1. The results (Fig. 5) indicated that pregrowth of the coli strain did not change the capacity of this sulfur-poor medium (some SO_4^{2-} is introduced as the iron salt) to support the growth of strain NT1. A similar procedure was followed for a culture of strain B with a low concentration of K_2SO_4. In this case, each increment of growth of the coli strain resulted in a proportional drop in the medium's capacity to produce a subsequent crop of the typhimurium strain (Fig. 5). These results indicate that the growth of *E. coli* B in glucose-MOPS medium occurs exclusively at the expense of the sulfate added to the medium until its concentration reaches a very low level. This suppression of MOPS degradation by sulfate is indicated also by the diauxic lag after sulfate exhaustion with either glucose (Fig. 4) or glycerol (results not shown) as carbon source.

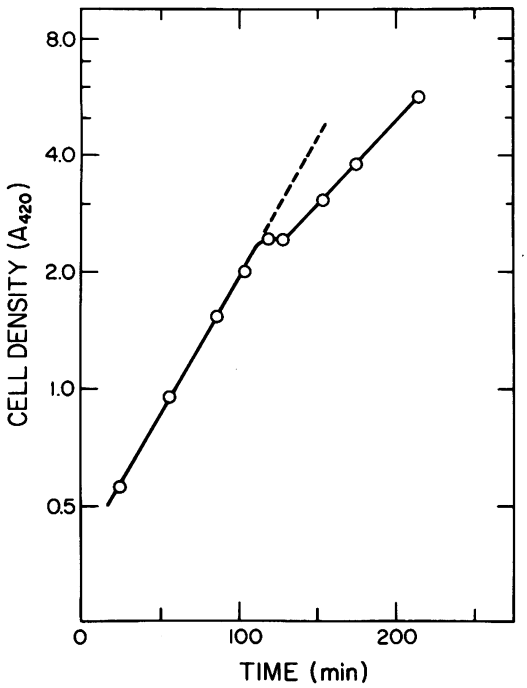

FIG. 4. *Growth of E. coli B in glucose-MOPS medium containing limiting sulfate. A flask containing 50 ml of glucose-MOPS medium with a reduced amount of K_2SO_4 (15 μM) was inoculated with E. coli B and incubated aerobically at 37 C. Growth was followed spectrophotometrically and is expressed as a logarithmic function of time.*

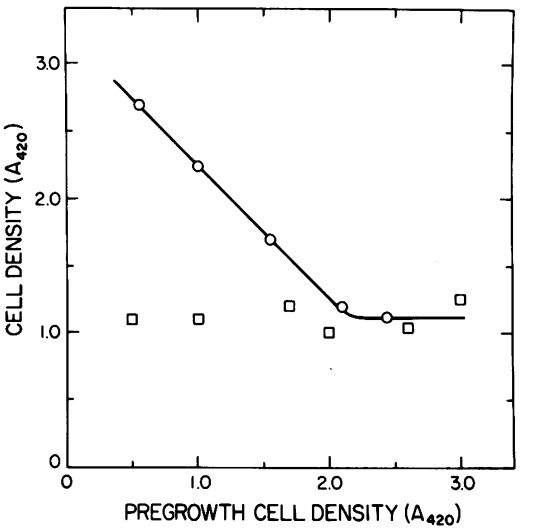

FIG. 5. *Effect of pregrowth of E. coli B on the ability of a sulfate-limited MOPS medium to support the growth of a culture of S. typhimurium NT1. A flask containing 50 ml of glucose-MOPS medium with no added K_2SO_4 (□) or with the K_2SO_4 concentration reduced to 15 μM (○) was inoculated with E. coli B at a low density (A_{420} = ~0.01) and incubated aerobically at 37 C. Samples were taken at several turbidities and chilled. The E. coli cells were removed by centrifugation followed by filtration, and then the sterile supernatant was inoculated at a low density with cells of S. typhimurium NT1 and incubated aerobically until growth stopped. The final cell density in each culture is plotted on the ordinate as a function of the E. coli density originally attained.*

Therefore, even for cultures of *E. coli* the MOPS medium can be used for sulfur-labeling experiments. Only for the purpose of sulfur starvation is the MOPS medium inappropriate for *E. coli*.

Nutrient requirements. Cultures of *S. typhimurium* were grown overnight in glucose-MOPS medium (glucose-limiting) and then inoculated into flasks of the same medium containing different amounts of a particular nutrient. Growth was carefully monitored, and the point at which growth decelerated was estimated from the growth curve. This point was defined as the growth-supporting capacity of that particular amount of nutrient. The results of those flasks in which the growth-supporting capacity was proportional to the amount of added nutrient were averaged (Table 2). The growth rates of the cultures were expressed as a function of initial nutrient concentration (Fig. 3). This procedure was sufficient to define the requirements for carbon and energy source (glucose), phosphate, sulfate, magnesium, and nitrogen (as ammonium ion).

For a number of components this procedure was inadequate to estimate precisely the nutritional requirement. The iron requirement of *S. typhimurium* NT1 growing on glucose is quite small and difficult to measure. Calculations of the possible iron contamination in the reagent chemicals used in the medium indicate a possible level of 2×10^{-4} mM, and without added iron a crop level of 1.5 to 2.0 A_{420} units is achieved with strain NT1. On the other hand, a requirement for additional iron is easily demonstrated with acetate as a carbon and energy source (Fig. 3) and probably reflects the greater role of respiration in acetate metabolism.

No effect of added calcium on either the growth rate or cell crop could be demonstrated (Fig. 3).

A number of trace elements are known to be required for the growth of fungi and to be present as prosthetic groups on one or another bacterial enzyme. The growth responses of *S. typhimurium* to various concentrations of copper, manganese, cobalt, molybdenum, boron, and zinc salts were tested. Only inhibitory effects were observed, and these occurred only at concentrations considered excessively high for such compounds. Copper toxicity was detectable in MOPS medium beginning approximately at 10^{-4} mM, cobalt inhibited growth at 2×10^{-1} mM, manganese slightly inhibited growth at 5×10^{-1} mM, and borate and molybdenum were just barely inhibitory at concentrations as high as 2 and 2×10^{-2} mM, respectively. No inhibition by zinc was observed as high as 6×10^{-2} mM (results not shown). Combined in the concentrations described by Machlis (8), these micronutrients had a slight effect on growth in MOPS medium (which of course contains a chelator) at the level used in fungal nutrition (Fig. 6). Interestingly, a mutant of *E. coli* B/r has been isolated in which the micronutrient components of MOPS medium are growth stimulatory (results not shown).

Salt concentration and ionic strength. Overnight cultures of *E. coli* B were prepared with glucose-limiting growth at an A_{420} of 1 and containing 0.15 mM NaCl. These cultures were used to inoculate flasks containing standard glucose-MOPS medium containing different amounts of NaCl at an initial A_{420} of 0.015. Growth was monitored in each flask, and the growth rates were expressed as a function of NaCl concentration (Fig. 7). The results indicate that NaCl slightly but significantly increases the growth rate of this *E. coli* strain up to a concentration of approximately 25 mM. Studies with *E. coli* B/r and *S. typhimurium* NT1 indicated an even smaller, and perhaps insignificant, effect on growth rate; for strain

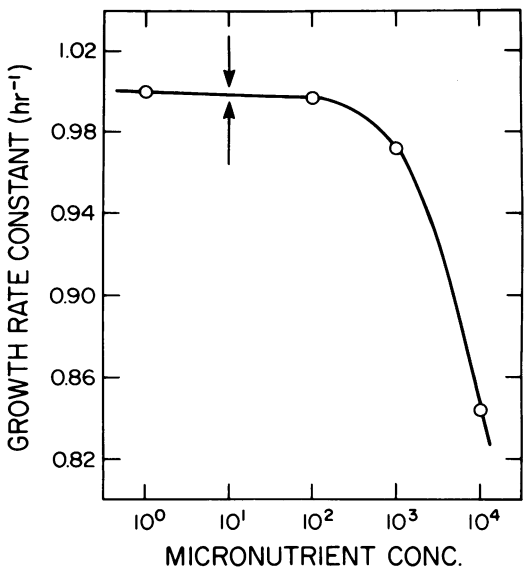

FIG. 6. *Effect of micronutrient concentration on the growth rate of S. typhimurium NT1 in glucose-MOPS medium. Growth rates are plotted on the ordinate as a function of the micronutrient concentration. The micronutrient concentration is plotted on the abscissa on a logarithmic scale. The concentration of micronutrients in the Machlis medium (8) is 10³. The arrows indicate the concentration chosen for normal MOPS medium, and the value for each component is listed in Table 1.*

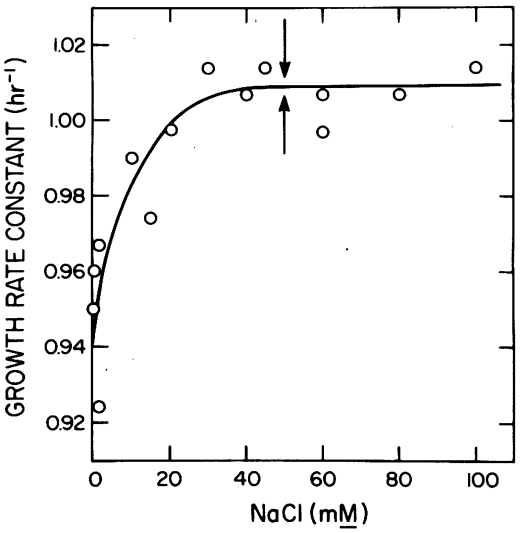

FIG. 7. *Effect of NaCl concentration on the growth rate of E. coli B in glucose-MOPS medium.*

NT1, for example, increasing the NaCl from 1.5 to 51.5 mM changed the average growth rate from $k = 0.99$ to 1.02. To examine whether this effect on growth rate in *E. coli* B was the result simply of the increased ionic strength provided by the added salt, the standard medium was varied in total concentration. At half the normal concentration the growth rate of strain NT1 was very slightly retarded ($k = 0.94$) compared with the normal concentration ($k = 1.0$); doubling the normal concentration had no significant effect. To examine whether the addition of 50 mM NaCl stimulated growth by supplying some needed element (other than sodium or chloride), small amounts of the most prevalent contaminants of reagent grade NaCl were tested for growth stimulation; neither KI, at 1.4×10^{-6} to 1.4×10^{-3} mM, nor KBr, at 1.8×10^{-7} to 1.8×10^{-4} mM, substituted for 50 mM NaCl. Finally, 584 g of NaCl was extracted with 600 ml of alcohol. The extract was evaporated to dryness, and the residue was dissolved in water. Addition of this extracted material did not effectively substitute for the NaCl supplement to the medium.

General properties of MOPS medium. Once formulated, the MOPS medium was examined for a number of properties considered important in bacterial physiology.

A culture of *E. coli* B/r was prepared in glucose-MOPS medium and maintained in exponential growth for several generations. When it reached an A_{420} of approximately 2, it was precisely diluted 25-fold into prewarmed media of the same composition. Growth was carefully monitored at 5-min intervals immediately after the dilution. No lag was detectable after the dilution (Fig. 8); a lag of as short as 30 s could have been detected. The same results were obtained in a separate experiment with *S. typhimurium* NT1.

Exponential-phase cultures of *E. coli* and *S. typhimurium* strains in glucose-MOPS medium were diluted serially to a final concentration of approximately 740 cells/ml in glucose-MOPS medium and then incubated under standard conditions. When the cultures became visibly turbid, their A_{420} was measured and the time was noted. From the dilution factor and the growth time, it was possible to calculate the overall growth rate of the cells for the whole incubation period. A more useful calculation, however, was the estimation of the overall lag period, assuming that the diluted culture experienced a lag period after which growth proceeded at the normal rate observed in turbid cultures (Table 4). *E. coli* B experienced a lag upon high dilution of approximately 5 h; *S. typhimurium* NT1 appeared to have a slight negative lag, probably the result of a slight difference between the real and the assumed

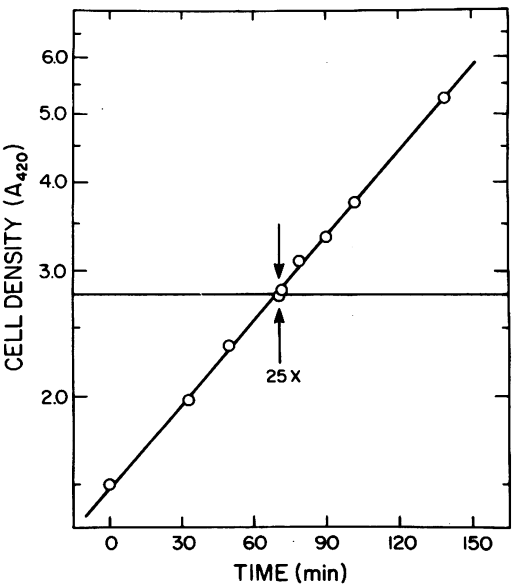

FIG. 8. *Effect of subculture dilution on the growth of E. coli B/r in glucose-MOPS medium. At the time indicated by the arrows, the culture was precisely diluted 25-fold into prewarmed glucose-MOPS medium; the cell density (A_{420}) shown on the ordinate has been multiplied by 25 for the diluted culture.*

TABLE 4. *Effect of dilution on growth*

Strain	Additions	Growth rate, k (h^{-1})	Dilution factor	Calculated lag (h)	
				Open flask	Closed flask
B	None	0.97	$\sim 10^6$	4.4	1.9
B	$NaHCO_3$ (0.1 mM)	0.96	2.6×10^6	3.4	1.0
B	$NaHCO_3$ (1.0 mM)	0.99	4.0×10^7	2.2	0.7
B	$NaHCO_3$ (10 mM)	0.98	$\sim 10^6$	0.0	0.0
B	None	0.97	4.0×10^7	6.1	4.3
B	$NaHCO_3$ (1.0 mM)	0.97	4.0×10^7	1.1	
B	Sodium succinate (.01%)	0.97	4.0×10^7	2.7	
NT1	None	0.89	2.0×10^6	−0.7	

growth rate for the cryptic period. These results were qualitatively quite reproducible: *E. coli* strains exhibited lags of 4 to 7 h, whereas *S. typhimurium* NT1 behaved as though unaffected by high dilution in glucose-MOPS medium. A number of measures were tested to eliminate the lag of *E. coli* strains, and two were found to be effective. If the dilution resulted in approximately 50 or more cells being present per ml, then sealing the flask to prevent exchange of gas with the environment reduced the lag of *E. coli* B to 1 to 2 h. This procedure was not effective if the initial cell density was 10-fold lower. A more effective measure was the addition of bicarbonate to the diluted culture. The addition of 10 mM $NaHCO_3$ virtually eliminated the lag period (Table 4). These results are interpretable if one assumes that there exists a low but critical value of pCO_2 for *E. coli* (1), and that at high cell densities glucose cultures easily maintain this by endogenous production. Consistent with this interpretation is the fact that succinate, which can bypass the CO_2 requirement for many cells, also reduces the lag period (Table 4).

Once the composition of the medium had been largely established, records were kept of the growth behavior of *E. coli* B/r and *S. typhimurium* NT1 over the course of several months of experimentation. For such measurements the cultures were sampled approximately 10 times between an A_{420} of 0.1 and 1.0, fixed, and diluted as described in Materials and Methods. Pregrowth for at least three generations was always permitted before the measurements commenced. The results of 30 experiments with triplicate cultures and 10 with duplicate cultures, with an average k of 1.00, were used to calculate a standard deviation of 0.0168 h^{-1}. By Student's t test, it was determined that a difference of 1.4 min per generation could be distinguished at a 95% confidence level between two sets of duplicate cultures. A separate investigator in our laboratory retrospectively examined the growth rates he had obtained with different batches of this medium over a 6-month period; 40 cultures of *S. typhimurium* NT1 in glucose-MOPS had an average k of 1.006 and a standard deviation of 0.030 in experiments that were not specifically designed to test how reproducible a rate could be achieved.

A number of media commonly used in metabolic and physiological studies with enteric bacteria were compared with the new MOPS standard medium. Table 1 lists the composition of these media, and Table 5 presents the results of growth experiments performed with *S. typhimurium* NT1. Comparable results were obtained with many strains of *E. coli;* growth rates were higher in glucose-MOPS medium than in other media, and for some *E. coli* strains the differences were quite marked. (The data of Table 5 indicate, incidentally, that there may be an advantage to filter sterilization for media using tris(hydroxymethyl)aminomethane (Tris) as a buffer.) Replacing MOPS buffer (40 mM) with Tris (10 mM) in the new medium caused a lowering of growth rate. In another experiment (not shown), replacing MOPS buffer with Tris at 40 mM caused little or no inhibition of growth of this strain of *S. typhimurium;* although pH buffering is not adequate at this concentration of Tris, it may be useful for work with low cell densities, particularly for such specialized purposes as sulfate limitation in *E. coli*. The main result shown in Table 5 is that no minimal medium tested is superior to the MOPS medium developed here.

The development of supplement media for physiological studies presents a separate nutritional task for each organism used. We did not make extensive tests on supplemented media, but determined that *E. coli* B/r and *S. typhimurium* NT1 could grow with a generation time of approximately 21 min at 37 C in a completely synthetic medium consisting of glucose-MOPS

TABLE 5. *Growth rate of S. typhimurium NT1 in various media*

Glucose medium[a]	No. of cultures	Growth rate constant, k (h^{-1})
Vogel-Bonner	1	0.937
Davis-Mingioli	2	0.961 ± 0.001[b]
Minimal A	2	0.962 ± 0.005
M63	2	0.968 ± 0.002
Modified Werkman	2	0.976 ± 0.003
M9	3	0.986 ± 0.002
M9 (filtered)	2	0.973 ± 0.040
Tris	2	0.687 ± 0.016
Tris (filtered)	2	0.724 ± 0.002
Tris-MOPS (filtered)[c]	2	0.824 ± 0.011
MOPS (filtered)	7	1.006 ± 0.005

[a] Composition of these media and references to the literature are given in Table 1. All media were autoclaved (without glucose), except those indicated as being filtered.

[b] The growth rate constant values are given with the standard error of the mean.

[c] The standard MOPS medium of Table 1 was changed by substituting Tris (0.1 M, pH 7.5) for MOPS buffer.

minimal medium to which was added the following L-amino acids (numbers indicate millimolarity): alanine (0.8), arginine (5.2), asparagine (0.4), aspartate (0.4), cysteine (0.1), glutamic acid (0.6), glutamine (0.6), glycine (0.8), histidine (0.2), isoleucine (0.4), leucine (0.8), lysine (0.4), methionine (0.2), phenylalanine (0.4), proline (0.4), serine (10.0), threonine (0.4), tryptophan (0.1), tyrosine (0.2), valine (0.6), adenine, guanine, cytosine, and uracil (0.2 each), and thiamine, calcium pantothenate, *p*-aminobenzoic acid, *p*-hydroxybenzoic acid, and 2,3-dihydroxybenzoic acid (0.01 each). Since this growth rate is indistinguishable from that obtained with various hydrolyzed digests of natural products commonly used for rich media, MOPS medium provides a good base from which to construct a variety of media permitting the whole range of growth states to be explored.

DISCUSSION

General objectives. Our goal was to prepare a medium that would be physiologically optimal for enterobacteria, experimentally useful for many nutritional, metabolic, and physiological purposes, and sufficiently convenient to attract wide usage.

Specifically, a medium with the following characteristics was desired: (i) the concentration of each of the major nutrient elements (phosphorus, nitrogen, carbon, and sulfur) should be independently adjustable and should be set at levels commensurate with its incorporation into bacterial protoplasm in order to facilitate isotopic labeling; (ii) the growth rate supported by the medium should be at least as fast as that supported by any known minimal medium; (iii) the medium should support growth up to cell densities that are useful for biochemical measurements; (iv) it should be easily constructed and inexpensive; (v) it should be stable, and preferably capable of being stored in concentrated form; (vi) it should give reproducible growth rates; (vii) it should permit absorbance measurements; (viii) growth of the cells should be supported indefinitely and should not depend on the adventitious presence of micronutrients; and (ix) the hydrogen ion concentration should be adequately buffered.

The MOPS medium described here meets all these specifications. A few comments about individual features of the medium are in order.

pH buffer system. In many respects the choice of a suitable buffer is the central issue in formulating a synthetic medium for biochemical studies on bacteria or other microorganisms. The physiologist's desire to achieve a high cell density places heavy demands on the pH buffering capacity of the medium and requires that the concentrations of certain ions, particularly $Fe^{2,3+}$, be near their solubility limit at neutral pH. Phosphate salts have been the classical choice for buffer because of a favorable pK and because the phosphate ion, as a metal chelator, provides a useful soluble reservoir of metal ions as well as an adventitious source of various elements required in trace amounts. Unfortunately, the usefulness of $^{32}P[PO_4]$ labeling in molecular biology requires that the phosphate concentration be reduced below the level at which it is an effective hydrogen ion buffer. The usual substitute has been Tris, but for many strains this substance is toxic at the high concentrations required by its unfavorable pK.

MOPS, one of a series of organic buffers devised by Good and his associates (5, 6) for use in biological work, has been chosen in place of Tris chiefly because of its useful pK (7.2 at 20 C), but also because it gave promise of not being toxic (3) and was relatively inexpensive. At concentrations at which it is a satisfactory buffer it has no detectable effect on growth. The formulation finally chosen (40 mM; initial medium pH of 7.2 at 37 C) supports growth on glucose throughout the recommended range of cell densities for critical physiological studies (up to an A_{420} of 2.0) with a drop of only 0.2 pH units, and will permit exponential growth up to

an A_{420} of 5.0 with a drop of only 0.5 pH units. Since glucose is the most acidogenic substrate known for enterobacteria, the medium should be satisfactory whatever the organic constituents of the medium.

MOPS cannot be used as either a carbon, nitrogen, or sulfur source by *S. typhimurium*. The strains of *E. coli* we tested cannot use MOPS as carbon or nitrogen source, but in the absence of added sulfate can use MOPS as a sulfur source. This utilization is completely suppressed by low levels of free sulfate, and therefore isotopic labeling with $^{35}S[SO_4^{2-}]$ presents no difficulty. The only procedure for which MOPS medium cannot be used is sulfur-restricted growth of *E. coli*. For this single purpose Tris can be substituted for MOPS, provided the pH is carefully monitored.

MOPS is a poor metal ion chelator and therefore has the desirable feature of being unlikely to introduce extraneous metal contaminants into the medium. At the same time, it is not helpful in maintaining a suitable $Fe^{2,3+}$ concentration at neutral pH. Tricine (6) does chelate $Fe^{2,3+}$, and the addition of a small amount of this organic buffer not only provides a satisfactory reservoir of soluble iron in the normal medium, but even permits the construction of a 10-fold concentrated medium for convenient storage.

Carbon and energy source. The use of glucose as a substrate for the growth of enterobacteria is so widespread that, despite the several unusual features of its metabolism by aerobic fermentation, it was chosen as a standard carbon and energy source, and the MOPS medium was developed largely with it. We specified two concentrations; the higher one is theoretically capable of supporting growth to a cell density of 1.5 mg (dry weight) per ml, which is fivefold above the maximum (A_{420} = 2.0; 0.286 mg [dry weight]/ml) recommended for physiological studies. This level is saturating for growth rate. The lower level was chosen to prepare overnight cultures; it supports exponential growth to an optical density of 1.05 (0.15 mg [dry weight] of cell/ml). Cells from these glucose-limited cultures are a convenient inoculum for most experiments as long as a suitable period of growth is permitted (preferably three generations) before measurements commence. Alternatively, in many cases it is preferable to inoculate overnight cultures with cells that are already in exponential growth; the size of the inoculum can be adjusted so that at a specified time the next day the culture will be at a desired density.

Macronutrients. The phosphate and sulfate in the new medium were set, like glucose at the levels equivalent to a cell crop of 1.50 mg (dry weight) per ml (A_{420} = 10.5), calculated from nutritional studies with *S. typhimurium* NT1. For all practical purposes these concentrations are saturating with respect to growth rate; also, the level of sulfate is sufficient to suppress MOPS degradation by *E. coli*. The magnesium level had to be set at a threefold higher concentration because it was not sufficiently clear that the level equivalent to a cell crop of 1.50 mg/ml was saturating with respect to growth rate (Fig. 3). Although determined for *S. typhimurium*, the levels of these components may be considered to be balanced for all practical purposes for *E. coli* as well. The particular salts used (K_2HPO_4, K_2SO_4, and $MgCl_2$) were chosen because of their ready availability, their use of either potassium or chloride as a counterion, and their relatively desirable characteristics with respect to stability, solubility, deliquescence, and presence of contaminants.

The same features hold for the nitrogen source chosen, NH_4Cl. The latter has been deliberately set at a level equivalent to a cell crop of 1.0 mg/ml (A_{420} = 7.0) so that, in the presence of excess carbon substrate, cultures in this medium all reach stationary phase at the same density and with the same limitation.

Iron. A requirement for added iron is easily demonstrated with substrates such as acetate that are metabolized largely by oxidative routes using iron proteins, and less easily with glycerol or glucose. With some media containing high concentrations of phosphate salts (Davis-Mingioli, M9, and Werkman, for example), an iron requirement is less easily demonstrated, presumably because of the introduction of iron as an adventitious contaminant. We set the iron level in MOPS medium at 10^{-2} mM because it is adequately saturating for such commonly used carbon sources as glucose and glycerol, yet is not detectably colored. For growth on acetate (and presumably succinate), the iron content should be increased fivefold (Fig. 3). At this level the iron will be growth-rate saturating and the medium will have only a faint yellowish tinge (A_{420} = 0.012); an alternative course—lowering the amount of chelating Tricine—is not recommended because of an unexpectedly narrow optimum range of iron concentration under this condition. We have found the heptahydrate of $FeSO_4$ to be a particularly convenient salt to handle. The small amount of sulfate (less than 4%) that this adds to the sulfate supplied as K_2SO_4 is only a minor violation of

our principle of using single sources of the main nutrients; if special circumstances require it, $FeCl_2$ can be easily substituted.

Micronutrients. In the absence of sensitive methods to determine the quantitative requirements of enterobacteria for copper, manganese, cobalt, molybdenum, boron, and zinc, it was necessary to adopt a different strategy. The components of a micronutrient solution (Machlis) that was developed to support the growth of fungi were each tested for toxicity to our strains of enterobacteria. Inhibitory effects were seen only at 1,000-fold higher concentration than present in Machlis solution. With safety it was possible, therefore, to specify a 100-fold less concentration for MOPS medium to help assure that the bacterial cells would be saturated for these elements and that fluctuations from laboratory to laboratory in the contamination levels of the other components of the medium (including the glassware and the water) would be rendered insignificant. As mentioned, the subsequent discovery of a mutant which responds to the micronutrient supplement of MOPS medium confirms the requirement for such elements and justifies their deliberate inclusion.

Salt concentration. The inclusion of 50 mM NaCl in the final formulation was done reluctantly. We attempted to keep the concentration of each component of the medium at the lowest level compatible with an optimum growth rate and a theoretical cell crop of 1.50 mg (dry weight) per ml. This conservatism was based on the desire to minimize the opportunities for adventitious introduction of stimulatory or inhibitory substances. There is no known requirement of bacteria for sodium, and chloride is already present in the medium in what would seem to be an adequate amount. No indication could be found that the slight but real stimulation of growth by 50 mM NaCl was the result of contaminants. Tentatively we conclude that it simply contributes to the ionic strength of the medium. Should the use of a medium totally free of sodium be desired, KCl could undoubtedly be substituted for the NaCl we arbitrarily chose.

Dissolved gases. This medium was developed by using aerobic growth as a determining guide. It does, of course, support anaerobic growth, but we made no extensive measurements on its characteristics under these conditions. The rotary-shaking Erlenmeyer flasks we used provided good aeration, but the difficulty of meeting the oxygen demand of rapidly growing, turbid bacterial cultures is well known. For this reason we have defined the upper limit of cell density in physiological studies with this medium as an A_{420} of 2.0 (just under 0.3 mg [dry weight] of cell/ml). We considered and rejected the use of forced aeration (sparging) because of its inconvenience, the difficulty of standardizing the composition of the gas from laboratory to laboratory, the likelihood of introducing chemical contaminants as gas-borne pollutants, and the problems created by the CO_2 requirement of the cells growing in glucose minimal medium.

The latter issue seems to be at the heart of the behavior of *E. coli* upon high dilution into glucose-MOPS medium. The several-hour-long lag periods under these conditions can, for all practical purposes, be eliminated by including 10 mM $NaHCO_3$ in the medium. This result is consistent with earlier reports of a CO_2 requirement for *E. coli* growing aerobically in glucose minimal medium (1). The problem is less acute with *S. typhimurium* NT1, and including $NaHCO_3$ seems unnecessary. It should be pointed out that there are at least two practical reasons for wanting to be able to dilute cultures down to a few cells per milliliter without introducing a lag period. First, the technique is useful, in the absence of a continuous automatic dilution device, to maintain cells in balanced growth for extensive periods, and, second, the excellent predictability of cell growth makes it possible to prepare cultures a day in advance and have exponential-phase cells at a particular density ready at a prespecified time the next day.

Convenience. The most convenient feature of the new medium is that it can be prepared and stored as a 10-fold concentrate to which only the phosphate salt and a carbon source need be added on dilution. For many investigators, the least convenient features of the medium may be the necessity of adding macronutrients and the necessity for filter sterilization. Sterilization of any culture medium by autoclaving is not recommended because of the difficulty of achieving a reproducible product; autoclaving this particular medium is not possible because of the heat lability of the buffer. Filter sterilization, however, is so conveniently performed with the ready availability of presterilized membrane filters (and holders) that this requirement is not considered an overbearing one. Only the concentrated medium need be filter sterilized; autoclaved distilled water is routinely used for preparation of the medium. The care required to prepare this medium is really quite small considering the importance of this aspect of physiological studies.

ACKNOWLEDGMENTS

This investigation was supported by grant GB 26461 from the National Science Foundation.

We are grateful for the suggestions and aid provided by W. G. McKeever and B. Wanner. N. E. Good generously sent us information about the nature, preparation, and properties of MOPS before publication, and offered several useful suggestions.

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