

EFFECT OF CERTAIN ACIDS OF THE TRICARBOXYLIC ACID CYCLE ON THE GROWTH OF *ESCHERICHIA COLI*¹

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In the course of an investigation (Johnson and Cohn, 1950) on the effect of a series of compounds on the inhibition of growth of *Escherichia coli* in work on an inhibition assay for pantothenic acid, it was found that the compound L-malic acid, instead of being inhibitory, was highly stimulatory to the growth of this organism. It was felt worthwhile to study further the effect of malate and also that of other acids of the Krebs' tricarboxylic acid cycle on the growth of *E. coli*.

Caswell, Koditschek, and Hendlin (1949) have reported that fumarate plus ethyl sodium oxalacetate would replace a tomato juice growth factor for the Dorner strain of *Lactobacillus lactis*; Wieland *et al.* (1950) have found that optimal growth of *Lactobacillus bulgaricus*, strain O9, is obtained only when citrate is added in addition to fumarate and ethyl sodium oxalacetate; and Skeggs *et al.* (1950) found that malate, citrate, α -ketoglutarate, and other Krebs' cycle acids stimulated the growth of *Lactobacillus leichmanii*, strain 4797. This indicates that in these lactic organisms some Krebs' cycle acids are not synthesized rapidly enough for optimum growth. Our data indicate a similar situation with respect to *E. coli*, the apparent limiting acid in this case being malic.

EXPERIMENTAL METHODS AND RESULTS

Escherichia coli, strain 221, from the University of Illinois, Department of Bacteriology collection was used in this study. Washed cells from a 24-hour culture grown on "bacto-micro inoculum broth" were resuspended in sterile saline and diluted 1 ml to 10 ml. One drop of such a suspension was used to inoculate each tube. The composition of the basal media used (Shive and Macow, 1946; Anonymous, 1949) is given in table 1. In all cases 5 ml of the double strength media (as given in table 1) are used per tube and diluted with water or aqueous solutions of the compounds added, to give a final volume of 10 ml per tube. The simple and the complex media gave the same results and, therefore, will not be reported separately. In testing the effect of the various acids of the tricarboxylic acid cycle on the growth of the organism, two procedures were used. First, the various acids studied were added in addition to the basal medium, whereas in the second procedure they were added at the expense of an equal molar quantity of glucose in the basal medium. In all cases the pH of the final medium was adjusted to 6.8. The growth was determined by turbidity after 18 hours' incubation at 37 C, the turbidity being read as per cent transmission at 650 $m\mu$ in a Coleman Model 14

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Universal spectrophotometer. The tubes were read both against distilled water set at 100 and against a tube of the basal medium inoculated and incubated with the assay. The results are reported in the tables as per cent transmission against water, while in the charts these values have been converted to optical densities.

The growth obtained by the addition of various acids to the basal medium is plotted in figure 1. From this figure it can be seen that while malic acid was the most active, the majority of the acids tried increased growth over the basal medium. This increase in growth occurred under conditions which were already giving very excellent growth of *E. coli*, yet malate at the highest level practically

TABLE 1
Basal media for the growth of Escherichia coli

	AMOUNT PER 1,000 ML DOUBLE STRENGTH	
	Medium 1	Medium 2
NH ₄ Cl.....	10 g	10 g
Fe(NH ₄) ₂ (SO ₄) ₂ ·6H ₂ O.....	0.2 g	0.2 g
MgSO ₄ ·7H ₂ O.....	0.8 g	0.8 g
K ₂ HPO ₄	8 g	8 g
Na ₂ SO ₄	10 g	10 g
Glucose.....	20 g	20 g
Enzyme hydrolyzed casein.....	2 mg	—
DL-Glutamic acid.....	—	125 mg
DL-Serine.....	—	20 mg
Thymine.....	—	6 mg
Xanthine.....	—	20 mg
Inositol.....	—	2 mg
Pyridoxine·HCl.....	—	6 mg
Nicotinic acid.....	—	400 μg
Riboflavin.....	—	400 μg
Thiamine·HCl.....	—	400 μg
p-Aminobenzoic acid.....	—	20 μg
Folic acid.....	—	6 μg
Biotin.....	—	2 μg
Vitamin B ₁₂	—	50 mμg

doubled this amount of growth. The addition of pyruvate or of additional glucose did not increase the amount of growth of the organism.

In figure 2 are given the growth results obtained when the compounds were added replacing equal molar amounts of glucose in the basal medium. It can be seen that when the acids are added in this manner, malic and fumaric acids have a marked growth-stimulating effect, while citric acid is stimulatory at moderate levels.

In order to study the effect of malate on the rate of growth as well as on the final growth produced, two series of 4 tubes each were set up: one set with malate, the other without, as in the preceding experiment. The tubes were inoculated and incubated as before, but in this case the turbidity was read at 8, 12, 17, 33, and 42 hours. At 8 hours there was no significant difference in growth (88.1 and

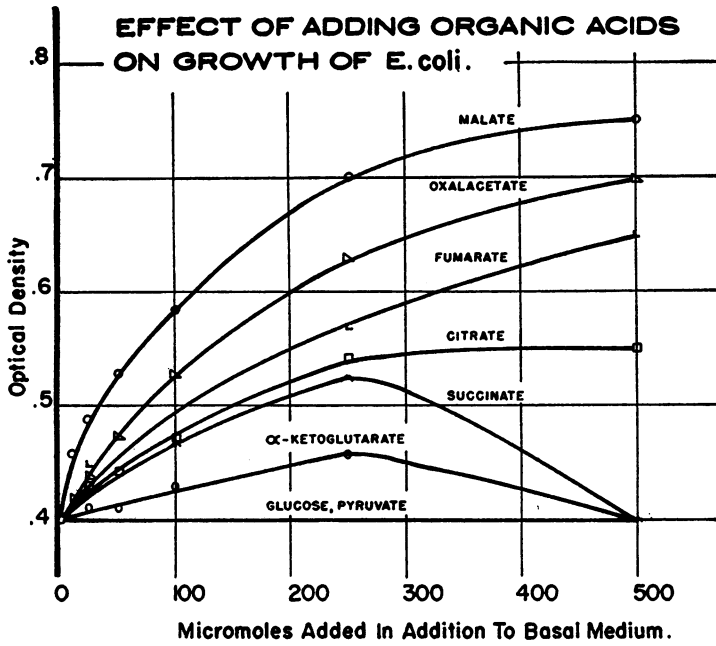


Figure 1. Effect of adding various organic acids to the medium on the growth of *Escherichia coli*.

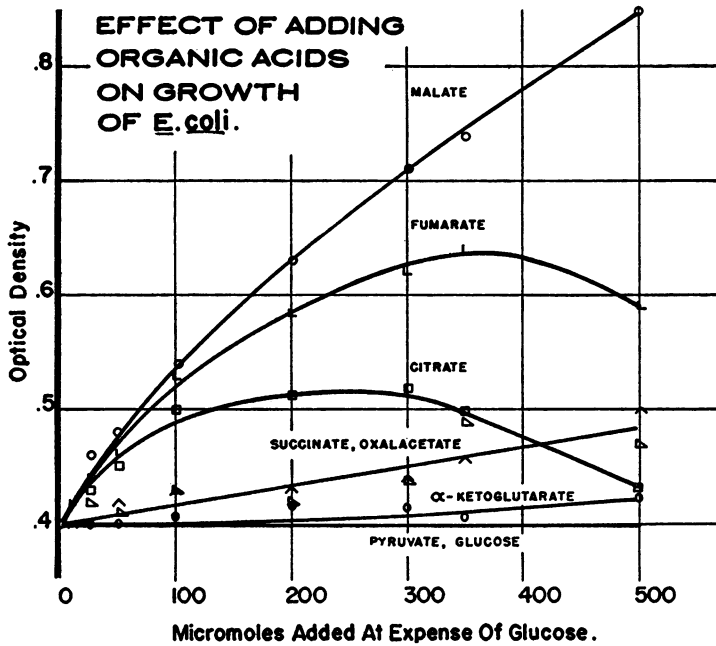


Figure 2. Effect of adding various organic acids to the medium, replacing equimolar amounts of glucose, on the growth of *Escherichia coli*.

88.8 per cent transmission). However, by 12 hours there was a great deal more growth in the tubes containing malate (29.2 per cent transmission compared to 50.5 per cent for the controls). After 17 hours there was no further increase in growth of the controls (39 per cent transmission at 17 hours and 38 per cent at 42 hours), while the tubes containing malate continued to grow during the whole period (17 per cent transmission at 17 hours and 7.5 per cent at 42 hours). From this it can be seen that the stimulatory effect of malate is on total growth rather than on early growth only.

The marked growth stimulation given by many of the acids added, and in particular by malate, in these experiments led to further experiments to try to elucidate the mechanism of the growth stimulation. Three possibilities primarily

TABLE 2
Effect of various organic buffers on growth and final pH

METABOLITE ADDED	AMOUNT, $\mu\text{M}/10$ ML	GROWTH, PER CENT TRANSMISSION	FINAL pH
Experiment 1			
None	—	43.0	4.4
Na acetate	500	39.5	5.6
Na malate	500	12.5	5.1
NH ₄ malate	500	20.0	5.1
Experiment 2			
None	500	35.0	4.8
Na acetate	500	36.0	5.7
Na citrate	500	30.0	6.1
Na succinate	500	25.0	6.0
Na malate	500	17.0	6.1
Experiment 3			
NH ₄ malate	500	17.0	6.1

Experiment 1. Basal medium contains 40 mg K₂HPO₄ and 600 μM glucose per 10 ml.

Experiments 2 and 3. Basal medium contains 40 mg K₂HPO₄ and 100 μM glucose per 10 ml.

were considered: (a) the malate served as a more available energy source than glucose; (b) the malate and other organic acid salts served as buffers to permit further growth; and, (c) the sodium malate (and other sodium salts, since the acids were neutralized with sodium hydroxide in all cases) served as a "dynamic buffer", the sodium ion remaining after fermentation of the malate being used to neutralize acids produced. All remaining experiments were conducted with medium 2, in some cases modified as to glucose and K₂HPO₄ concentration as described in the text.

The effect of the various sodium salts as buffers is reported in table 2. From this table it can be seen that sodium malate did not function chiefly as a buffer, since sodium salts of other organic acids which were equally successful in maintaining the pH had little to no growth-promoting activity. To test the functioning of sodium malate as a "dynamic buffer" ammonium malate was used both in

addition to, and in substitution for, glucose. In this test the NH_4Cl was omitted from the basal medium, the ammonium malate supplying the same amount of nitrogen. The results are included in table 2. It appears that sodium malate and ammonium malate were approximately equally active in increasing the growth of *E. coli*.

To study further the effect of buffers, increasing levels of K_2HPO_4 from 0 to 100 mg (0 to $570 \mu\text{M}$) per tube were added to phosphate-free medium. The effects on the growth of *E. coli* and on the final pH values are plotted in figure 3. In addi-

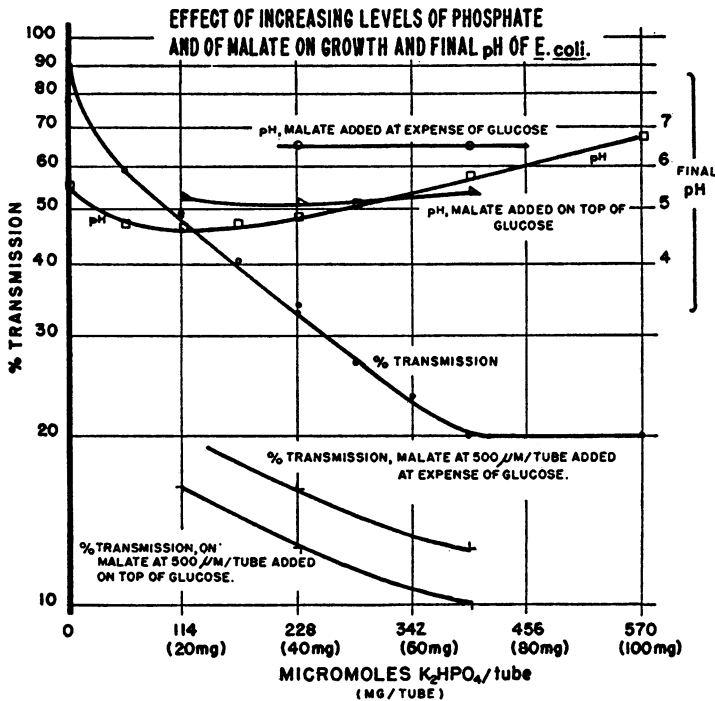


Figure 3. Effect of increasing levels of phosphate and of malate on the growth of *Escherichia coli* and on the final pH of the culture. The graphs labeled per cent transmission and pH record the effect of increasing the level of phosphate in the basal medium, while the other graphs record the effect of malate added at different phosphate levels.

tion, the growth data and final pH values obtained when malate (67 mg, $500 \mu\text{M}$ per tube) was added, both in addition to, and in substitution for, the glucose of the medium at different phosphate levels are plotted in this figure.

From these graphs it can be seen that there is a marked growth stimulation on increasing the phosphate level to 70 mg ($399 \mu\text{M}$) per tube but that in all cases malate addition gave more growth.

To try to separate the phosphate requirement from the phosphate buffer effect, the organic forms of phosphate, fructose-6-phosphate, hexose diphosphate, and adenosinetriphosphate were examined for their effects on growth and final pH.

The effects of fructose-6-phosphate are given in table 3. (The results with hexose diphosphate or adenosinetriphosphate were similar.) From this table it can be seen that on a molar basis compared to K_2HPO_4 , fructose-6-phosphate produced only slightly less growth but a lower pH due to less buffering.

Since malate so greatly stimulates the growth of *E. coli* in the presence of glucose, malate was tried as sole energy source either as sodium or ammonium malate. In both cases at 500 μM (equivalent to 67 mg malic acid per tube) much less growth (75 and 73 per cent transmission) was obtained than with glucose. Apparently some glucose is required to initiate the fermentation, serving as primary hydrogen donor. In order to study this relationship, the growth and final pH of

TABLE 3
Fructose-6-phosphate as PO_4 source added to phosphate-free medium

AMOUNT/10 ML		GROWTH, PER CENT TRANSMISSION	FINAL pH
Mg	μM		
0	0.0	96.0	5.2
10	36.2	78.0	4.3
20	72.4	64.0	4.7
50	181.0	41.5	4.7

TABLE 4
Glucose as a "starter" for the fermentation of malate

AMOUNT GLUCOSE, μM /10 ML	NO MALATE ADDED		MALATE ADDED, 500 μM /TUBE	
	Growth % transmission	Final pH	Growth, % transmission	Final pH
0	100	6.8	75.0	7.3
10	70	7.2	56.0	7.2
20	55	7.0	43.0	7.0
50	36	6.7	24.0	6.8
80	27	6.5	16.0	6.6
100	24	6.2	12.5	6.5
600	22	5.7	10.0	5.3

E. coli grown in the presence of decreasing levels of glucose with and without added malate were determined. This experiment was carried out in the presence of optimum K_2HPO_4 so that final pH would not be the limiting factor on growth. The results are given in table 4. From this table it is apparent that on this medium excellent growth occurs with 18 mg glucose (100 μM per tube) per 10 ml and that little further growth but increased acid production results upon increasing the glucose from 18 to 100 mg per 10 ml (600 μM per tube). In all cases the addition of malate markedly improves growth with no effect on the final pH.

Since a function of malate appeared to be that of hydrogen acceptor, acting in lieu of oxygen, an experiment was conducted to compare the effect of malate with that of aeration on the growth of *E. coli*. Half of the assay was set up in 125

ml Erlenmeyer flasks with mechanical shaking during the 16-hour incubation period while a duplicate assay was carried out simultaneously in test tubes without shaking. As before, 10 ml volumes were used in both cases.

The results of this experiment are reported in table 5. From this table it can be seen that actually malate was more stimulatory in shaken flasks than in the test tube assay. On the basis of 100 per cent transmission for glucose alone (i.e., setting the 0-malate-no-shaking tube, which read 42 against water, at 100), the transmission reading with malate is 45; while in the Erlenmeyers (when the 0-malate-with-shaking flask, which read 15.5 against water, is set at 100) the malate-added flask reads 13.

A further experiment was carried out in which the amount of growth was determined under anaerobic conditions (paraffin-sealed tubes). In this case again, malate markedly increased growth. At 18 hours the per cent transmission was 41

TABLE 5
Effect of aeration and of malate on growth of Escherichia coli

AMOUNT GLUCOSE, $\mu\text{M}/10\text{ ML}$	AMOUNT SODIUM MALATE, $\mu\text{M}/10\text{ ML}$	GROWTH, PER CENT TRANSMISSION	FINAL pH
In test tubes without shaking			
600	0	42.0	4.6
100	500	19.0	5.8
In Erlenmeyers with continuous shaking			
600	0	15.5	5.4
100	500	2.0	8.7

for the control tubes and 19 for the tubes containing malate (added at 500 μM per tube replacing 800 μM of glucose).

DISCUSSION

It appears that on the 1 per cent glucose medium used the growth of *E. coli* is somewhat limited by the phosphate level. However, it appears to be limited even more by the inability of the Krebs' cycle enzymatic system, or some part of this system, to provide some of the components of the tricarboxylic acid cycle in rates adequate for maximum growth. Thus, the addition of many of these acids in addition to the glucose of the medium increases growth by providing in excess these immediately utilizable compounds as energy sources. In contrast, the addition of extra glucose or pyruvate has no effect on the growth rate, showing, we believe, that the effect is primarily one of more readily available energy.

Whatever the reasons involved, it is apparent that increasing the phosphate level from 40 to 70 mg K_2HPO_4 per 10 ml (228 to 399 μM) and the addition of 350 to 500 μM of malate (47 to 67 mg malic acid) per 10 ml of medium give a basal medium which permits more than double the amount of growth previously obtained. This is proving of value in the use of this organism for assay work.

Since malic acid appeared to be such an efficient energy source for *E. coli*, its ability to promote growth in the rat was tested. However, rats pair-fed a ration containing 10 per cent malic acid did not gain at a rate significantly different than pair mates fed the same ration with the malic acid replaced by glucose.

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SUMMARY

Malic and fumaric acids when added to a "complete" medium for *Escherichia coli* at levels up to 500 μM per 10 ml of media were found to markedly stimulate the growth of the organism. At 500 μM per 10 ml of media, malic acid (neutralized with either NaOH or NH_4OH) approximately doubled the amount of growth obtainable.

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