AN ABSTRACT OF THE THESIS OF

MARK W. KRUK   for the degree of   MASTER OF SCIENCE

in Food Science and Technology presented on ___3/20/81___

Title: INHIBITION OF ESCHERICHIA COLI TRIMETHYLAMINE-N-OXIDE REDUCTASE

BY FOOD PRESERVATIVES

Abstract approved: _____

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Trimethylamine-N-oxide (TMA-O) reductase activity of resting cells

of Escherichia coli was inhibited by tetrasodium ethylenediaminetetraacetate

(Na_4EDTA), benzoic acid (BA), and methylparaben (MP). The 50% inhibitory

concentrations of Na_4EDTA, BA, and MP were 20.2, 1.2, and 32.4 mM,

respectively. BA at pH 6.5 or below most effectively inhibited the TMA-O

reductase.

Sorbic acid (SA), up to 0.70 mM, had no effect on TMA-O reductase

activity, but SA inhibited the growth and subsequent TMA production in

E. coli at or above 0.35 mM.

Inhibition of _Escherichia coli_ Trimethylamine-N-oxide

Reductase by Food Preservatives

by

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A THESIS

submitted to

Oregon State University

in partial fulfillment of

the requirements for the

degree of

Master of Science

June 1981

APPROVED:

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ACKNOWLEDGMENTS

I would like to dedicate this work to my wife, Debber, whose love and perseverance made it possible.

I would like to thank Dr. Jong S. Lee for his hours of assistance, patience and driving enthusiasm.

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INHIBITION OF ESCHERICHIA COLI TRIMETHYLAMINE-N-OXIDE REDUCTASE BY FOOD PRESERVATIVES

INTRODUCTION

The trimethylamine (TMA) content of marine fish has been commonly used as an index of spoilage (8, 18). TMA is produced from trimethylamine-N-oxide (TMA-O) present in fish muscle by TMA-O reductase specified by bacteria (12, 20, 21).

Many antimicrobial agents are known to inhibit TMA production in fish. Some of these are sodium chloride (11), sodium nitrite (6), benzoic acid (17), and sorbic acid (4). It has never been clear, however, whether this was directly due to inhibition of TMA-O reductase or was a result of reduced microbial growth.

This investigation was undertaken to determine the effects of sorbic acid (SA), benzoic acid (BA), methylparaben (MP), and tetrasodium ethylenediaminetetraacetate (Na_4EDTA) on the TMA-O reductase activity of resting cells of Escherichia coli.

MATERIALS AND METHODS

Microorganism

The IMViC typical Escherichia coli culture used was originally
isolated from a seafood and has been maintained as a stock reference
culture in this laboratory. Cells were grown statically in brain
heart infusion (BHI, Difco) broth at $37^{o}C$ for 24 h prior to use. All
tests were conducted at an incubation temperature of $37^{o}C$ under static
conditions. TMA production was measured by Dyer's picrate method (5),
modified by Murray and Gibson (13).

Growth and trimethylamine (TMA) production

Cells prepared as above were inoculated into four pairs of duplicate
250 ml screw cap bottles containing 100 ml BHI broth with 0.1% TMA-O
(Aldrich, Milwaukee, WI) and various levels of potassium sorbate
(Monsanto, St. Louis, MO). Samples were withdrawn at 0, 1, 3, 5, 8 and
12 day intervals for TMA determination and viable cell count.

TMA-O reductase activity

Cells were washed three times in sterile 0.067 M phosphate buffer
by centrifugation. A 50 ml aliquot of the cell suspension was added
to the basal medium (BM) which contained 50 ml phosphate buffer, 0.1%
TMA-O and 0.05 M glucose (Baker, Phillipsburg, NJ) in a 250 ml screw
cap bottle. Samples were removed at hourly intervals for TMA determination.
Protein content of the cell suspensions was measured by the Kjeldahl

method (10).

Inhibitors

Each culture bottle was also prepared with BM plus various
concentrations of potassium sorbate, sodium benzoate (Mallinckrodt,
St. Louis, MO), methylparaben (Washine Wks, Lodi, NJ) and tetrasodium
ethylenediaminetetraacetate (Matheson, Coleman, Bell, Norwood, OH).
Some BM without inhibitors were adjusted to pH 5.0, 6.0, 6.5, 7.0, 7.5
and 8.0 with 1.0N HCl and 0.1N KOH to determine the pH optimum for the
TMA-O reductase activity.

RESULTS AND DISCUSSIONS

Effect of preservatives on TMA-O reductase

TMA-O reductase activities of resting E. coli cells in the presence of the four preservatives are presented in Table 1. The effectiveness of the preservatives is calculated as an Inhibition Index (I), according to the formula proposed by Freese (9). I is defined as 1 - test value divided by that of the control. Complete inhibition would give a value of 1.0 and no inhibition would give a 0.0 value.

Benzoic acid (BA) at 2.5 mM level and pH reduced to 6.0 showed almost complete inhibition of the TMA-O reductase activity. At pH 6.5 and 0.81 mM BA, the inhibition was still greater than 50%. Eklund (7) showed that a much higher concentration of 13 mM of BA at pH 6.6 was necessary for 50% inhibition of E. coli growth. It appears, therefore, the effect of BA on the TMA-O reductase of E. coli is far greater than that on the cell growth.

Methylparaben (MP), up to 13.1 mM level, showed limited inhibition, i.e., I of 0.15. At 19.7 mM the I had more than doubled to 0.32.

Tetrasodium ethylenediaminetetraacetate (Na_4EDTA) completely inhibited TMA-O reductase activity at 34.2 mM concentration.

Sorbic acid (SA) had no inhibitory effect on TMA-O reductase activity at concentrations as high as 0.7 mM.

The TMA-O reductase activity of three controls run with preservatives, ranged from 0.51 to 0.72 $\frac{ug/ml}{h}$/mg protein. This variation could have been introduced by some differences in the levels of dissolved oxygen in the BM and the differences in the ambient temperatures of TMA

Table 1.   Trimethylamine-N-oxide (TMA-O) reductase activity of E. coli in the presence of preservatives.

Inhibitor[a]	Conc. (mM)	pH	Max. TMA Produced (ug/ml)	TMA-O Reductase Activity($\frac{ug/ml}{h}$/mg protein)	Inhibition[c] Index (I)
SA	0.00[b]	7.2	107.5	0.51	0.00
	0.17	"	110.0	0.38	0.25
	0.26	"	107.5	0.45	0.12
	0.35	"	105.0	0.46	0.10
	0.53	"	105.0	0.51	0.00
	0.70	"	97.5	0.51	0.00
Na$_4$EOTA	0.0	7.2	160.0	0.69	0.00
	3.4	"	147.0	0.60	0.13
	8.5	"	147.0	0.54	0.22
	17.1	"	105.0	0.41	0.41
	25.6	"	85.0	0.35	0.50
	34.2	"	2.7	0.01	0.99
MP	0.0[b]	7.2	132.0	0.72	0.00
	1.3	"	132.0	0.71	0.02
	6.6	"	125.0	0.69	0.05
	13.1	"	107.0	0.61	0.15
	19.7	"	92.0	0.49	0.32
BA	0.02[b]	8.0	109.0	0.42	-0.23[d]
	0.08	7.5	107.5	0.46	-0.15
	0.25	7.0	97.5	0.40	0.16
	0.81	6.5	39.0	0.14	0.57
	2.50	6.0	2.5	0.01	0.97

[a]SA = sorbic acid, Na$_4$EDTA = ethylenediaminetetraacetate, MP = methylparaben, BA = benzoic acid.

[b]Theoretical undissociated acid concentration.

[c]I = 1 - (test value/control value).

[d]I was calculated from control value at each pH.

extraction. Recently, Sakaguchi and Kawaii (15) have shown that oxygen inhibited the synthesis of TMA-O reductase and Bullard and Collins (1) identified the differences in the ambient temperature as a source of variation in Dyer's method for TMA (5). Since each preservative was tested under identical conditions with the respective controls, such variation should not diminish the comparative values presented in Table 1.

Relative effectiveness of preservatives

Figure 1 shows the linear regression lines for inhibition index (I) vs. inhibitor concentration. BA showed a good correlation of 0.93 with a slope of 0.45. Na_4EDTA showed better linearity with a correlation of 0.97, but the slope dropped to 0.03. At the 1.0 mM level, the percent inhibition by BA and Na_4EDTA was 39 and 2, respectively, indicating that BA was more inhibitory to TMA-O reductase.

Unemoto et. al. (19) reported that 1.0 mM Na_4EDTA inhibited a partially purified TMA-O reductase of Vibrio parahaemolyticus to 9% of its initial activity. The relative ineffectiveness of Na_4EDTA we observed, therefore, could be due to the intact cell.

MP showed a good correlation of 0.97 between I and inhibitor concentration, but the slope was only 0.02, indicating less effectiveness than Na_4EDTA. The limited solubility of MP (16.4 mM in H_2O at 25^oC) precluded investigation of this compound at higher concentrations (3).

SA showed a negative slope of -0.10 which indicated a possible stimulatory effect at these concentrations, but the poor negative correlation of $^-$0.37 made such interpretation uncertain. SA, at the levels tested, therefore, did not appear to inhibit the TMA-O reductase
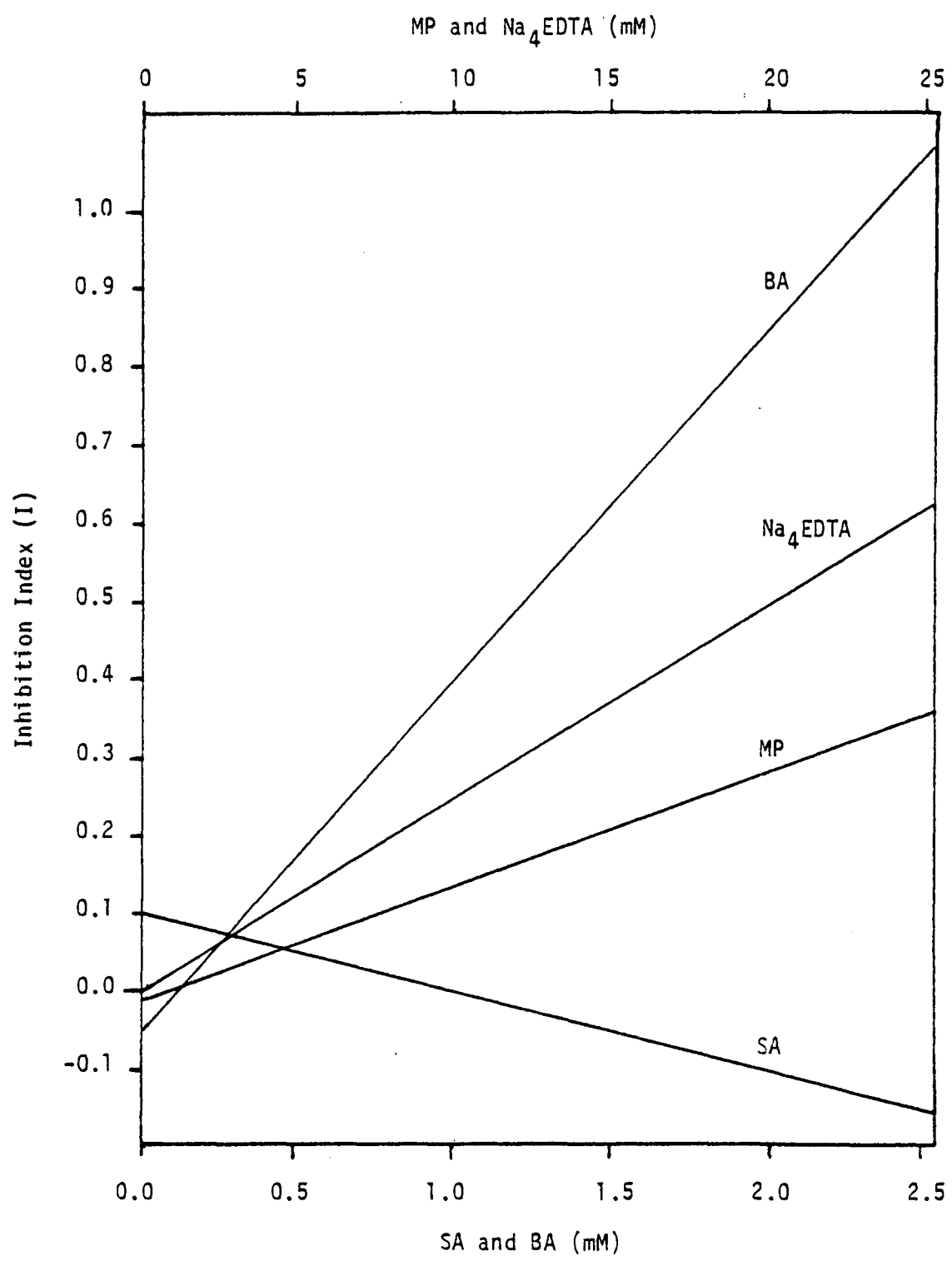
Figure 1. Linear regression lines for Inhibitor Index (I) vs.
preservative[a] concentration.

[a]BA = benzoic acid, Na₄EDTA = tetrasodium ethylenediamine-
tetraacetate, MP = methylparaben, and SA= sorbic acid.

activity of E. coli.

Effect of pH on TMA-O reductase

Figure 2 shows the TMA-O reductase activity of E coli resting cells measured at various pH. The maximum TMA-O reductase activity was at pH 7.0. This was slightly lower than the maximum of 7.5 to 7.8 reported by Castell and Snow (2). With BA, however, the optimum shifted toward alkaline pH and the inhibitory effect at low pH was far more pronounced. Thus, the strong inhibitory effect of BA on TMA-O reductase of E. coli was apparently due to a combination of lower pH plus the increased concentration of undissociated BA at such pH (3, 7, 16).

Effect of sorbic acid on growth and TMA production in E. coli

The growth and TMA production of E. coli in the presence of SA is presented in Figure 3. The TMA level of 122.5 ug/ml and the maximum log viable count of 9.04 were obtained in the controls. At 0.17 mM SA the maximum growth was reduced to 7.38, while the TMA production was only slightly reduced to 105.0 ug/ml. At SA concentrations of 0.35 and 0.70 mM the TMA levels and maximum cell growth were both drastically reduced. SA, therefore, appears to inhibit the growth of E. coli more than its TMA-O reductase activity and the reduction of TMA production in the presence of SA appears to be the indirect result of reduced microbial growth.
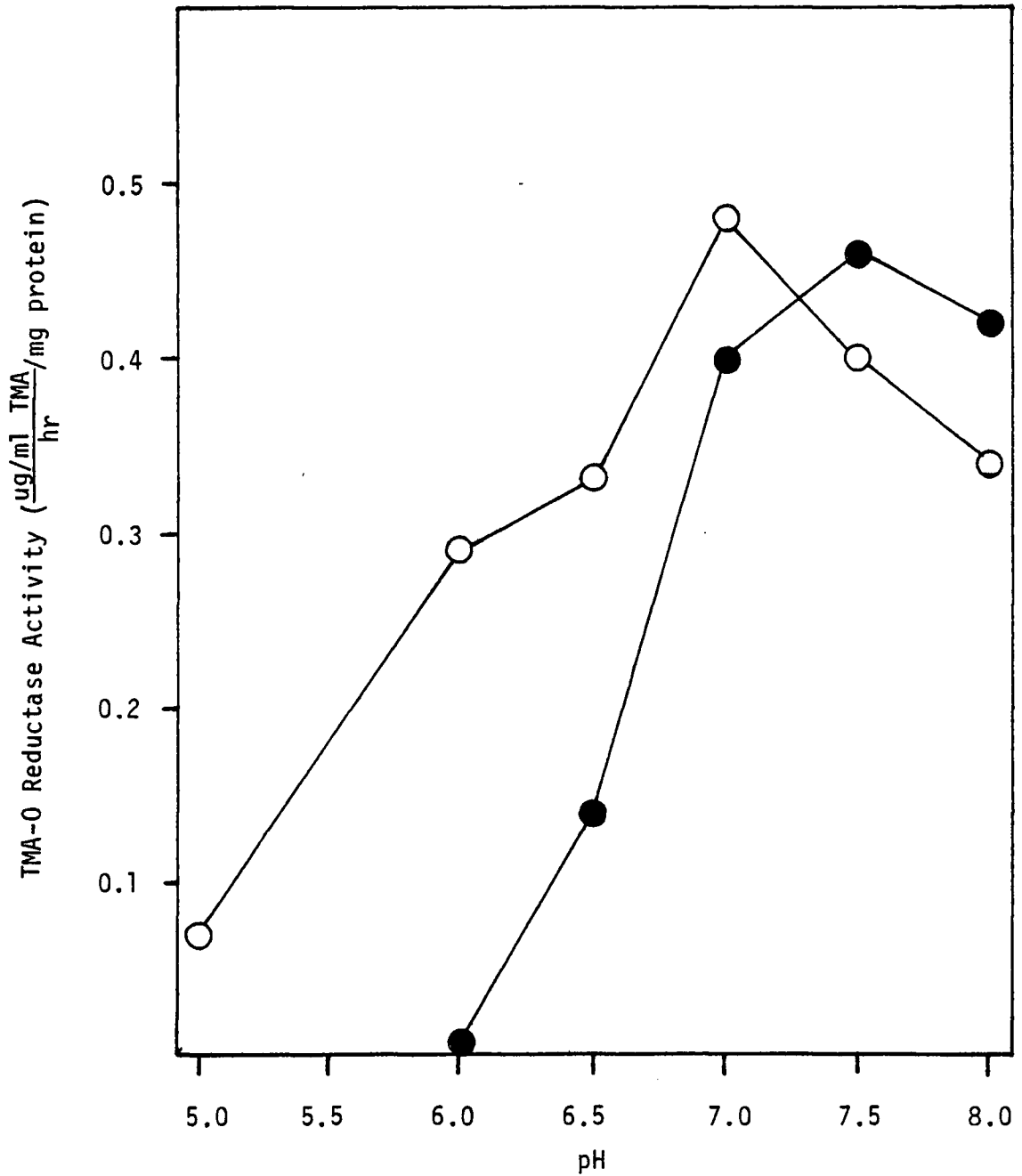
Figure 2. Effect of pH on TMA-O reductase activity of E. coli.[a]

[a] ○ = basal medium (BM) containing 50 ml phosphate buffer, 0.1% trimethylamine-N-oxide (TMA-O) and 0.05M glucose, ● = BM plus benzoic acid.
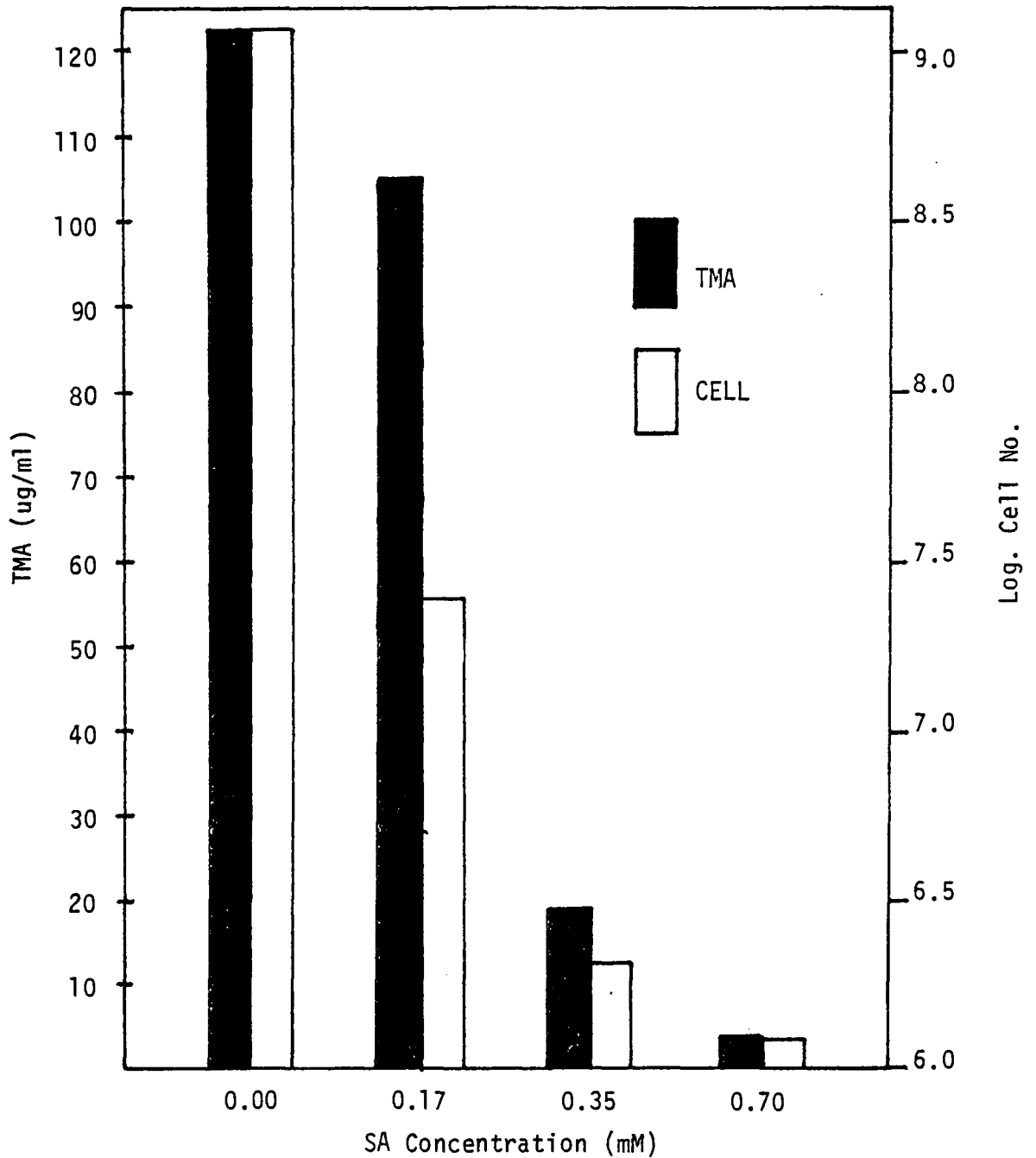
Figure 3. Effect of sorbic acid (SA) on the growth and trimethylamine (TMA) production of E. coli.

SUMMARY AND CONCLUSIONS

Among the four preservatives tested, BA was the most effective inhibitor of the TMA-O reductase. Na_4EDTA was equally effective as BA, but required a 10-fold increase in concentration. MP was only moderately effective in inhibiting the TMA-O reductase, due to low solubility. SA did not directly inhibit the TMA-O reductase, but it did suppress the TMA production by inhibiting microbial growth.

It appears that some preservatives not only inhibit the growth of microorganisms but also the enzymatic activity associated with spoilage. Inhibition of enzymatic activity independent of growth inhibition can be demonstrated with resting microbial cells since these cells are not reproducing.

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