Evaluation of Factors Affecting the Survival of *Escherichia coli* in Sea Water

VI. Cysteine[1]

P. V. Scarpino[2] and David Pramer

Department of Agricultural Microbiology, Rutgers, The State University, New Brunswick, New Jersey

Received for publication April 4, 1962

ABSTRACT

Scarpino, P. V. (Rutgers, The State University, New Brunswick, N.J.) and David Pramer. Evaluation of factors affecting survival of *Escherichia coli* in sea water. VI. Cysteine. Appl. Microbiol. **10**:436–440. 1962.—The relationship between death of cells of *Escherichia coli* in artificial sea water and time was established as linear, and statistical tests demonstrated that the most suitable measure of survival was log per cent after 24 hr. Survival of *E. coli* in water supplemented with cysteine at levels of 0.284×10^{-6} to 284×10^{-6} M was increased greatly over that in untreated water. To provide an insight into the mode of action of cysteine, the effect of concentration of various sulfhydryl and disulfide compounds was measured, and the influence of several compounds that lack a functional sulfur group but which are capable of affecting oxidation-reduction potential was determined. Moreover, a number of substances related structurally to cysteine were tested to ascertain their influence on the survival of cells of *E. coli* in artificial sea water. It appeared that the beneficial effect of cysteine was not due to the sulfhydryl group of the amino acid or to the ability of the compound to influence oxidation-reduction potential. Some sulfhydryl compounds had no favorable effect and, in general, disulfides were more active than the corresponding sulfhydryl compounds. Substances that lack a functional sulfur group but influence oxidation-reduction potential had no significant activity. The beneficial effect of a number of compounds related structurally to cysteine indicates that both an amino and carboxyl group are required for activity. It is suggested that cysteine and other amino acids act to increase survival of cells of *E. coli* in sea water by a chelation mechanism.

Johannesson (1957) reported that low levels of cysteine greatly increase survival of cells of *Escherichia coli* in sea water. He suggested that iodate is responsible for the rapid death of bacteria in a marine environment and that cysteine exerts a protective influence as a result of its reactivity with halates. Shaw and Cooper (1957) reviewed the mechanism of iodine oxidation in sea water and concluded that iodate is not a likely product. They suggested that the oxidized form of iodine present in sea water is an equilibrium mixture of iodide and hypoiodious acid. This view was challenged by Sugawara and Terada (1958) and Johannesson (1958) and remains to be clarified.

The beneficial effect of cysteine on the survival of *E. coli* in natural sea water was confirmed by Carlucci and Pramer (1960*b*), and the amino acid was demonstrated to have a similar effect in artificial sea water (Carlucci, Scarpino, and Pramer, 1961). This activity of cysteine was of sufficient interest to warrant further study, and the present report summarizes the results of a series of investigations using artificial sea water to provide an insight into the mechanism by which the amino acid acts to increase the survival of cells of *E. coli*.

Materials and Methods

Detailed descriptions of the methods employed for the preparation and use of inocula, treatment and storage of water samples, and of the enumeration of *E. coli* in sea water were presented previously (Carlucci and Pramer, 1960*a*). Artificial sea water was prepared according to MacLeod and Onofrey (1956). This formulation was demonstrated (Carlucci et al., 1961) to exert a marked bactericidal action that is offset by cysteine.

Curves depicting the death of cells of *E. coli* added to natural sea water collected off the New Jersey coast showed an initial lag phase of approximately 24 hr, a period of linear decline or logarithmic death lasting 48 to 72 hr, and, finally, an equilibrium phase in which the curve became asymptotic (Carlucci and Pramer, 1960*a*). Therefore, previous papers of this series reported survival in terms of per cent after 48 hr. This terminal period was on the linear portion of the curves and provided a convenient measure of the influence of various factors on the survival of cells of *E. coli* in sea water (Carlucci and Pramer, 1960*b*, *c*, *d*; Carlucci et al., 1961). Curves obtained by the periodic enumeration of cells of *E. coli* added to artificial sea water differed from those obtained with

[1] Paper of the Journal Series, New Jersey Agricultural Experiment Station, New Brunswick, N.J.

[2] Present address: Fairleigh Dickinson College, Rutherford, N.J.

natural sea water in that no lag period preceded logarithmic death. Statistical analyses using Bartlett's test, as described by Dixon and Massey (1957), demonstrated that the variances of log per cent survival values were homogeneous, whereas those for per cent survival were not. Accordingly, measurements of survival of *E. coli* in artificial sea water are reported and compared as log per cent survival, rather than per cent survival, at the end of 24 hr. The smaller or more negative the value, the more rapid was the decrease in number of viable cells.

To establish the relationship between time and survival of cells of *E. coli* in artificial sea water, 36 flasks, each containing 100 ml of artificial sea water, were inoculated equally with a suspension of cells of the test organism. The flasks were incubated at 28 C without agitation, and survival was measured in triplicate at 2-hr intervals for 24 hr, using separate flasks at each test period. Regression analysis showed that the lack of fit of the data to a linear model was not significant at the 95% confidence level, and it was concluded that a straight line adequately described the relation between survival of cells of *E. coli* in artificial sea water and time. The calculated regression equation was $\log y = 6.47747 - 0.12675\,x$, where y is the number of viable bacteria/ml and x is the time in hr. The fit of this line to the experimental data is illustrated in Fig. 1.

The t statistic was used to calculate values for the required difference between two means for significance at a probability level of 95%. The equation employed was:

$$x_1 - x_2 \pm ts\sqrt{\frac{1}{n_1} + \frac{1}{n_2}}$$

where x_1 is the mean of sample 1; x_2 is the mean of sample 2; n_1 is the number of observations in x_1; n_2 is the number of observations in x_2; t is the value at the desired confidence level; and s is the standard deviation. Since the present investigations employed single observations only, $n_1 = n_2 = 1$, and solution of the above expression yielded a value of 0.622 for the minimal difference that had to be obtained between two log per cent values for significance at a confidence level of 95%. It was assumed that the variances determined for the experimental results summarized in Fig. 1 applied throughout these investigations, and the calculated difference between means (0.622 log units) required for significance at the 95% level of probability was applied generally.

Results and Discussion

The influence of concentration of cysteine on survival of cells of *E. coli* in artificial sea water is evident from the results listed in Table 1. The bacterium benefited significantly (P = 0.05) from 0.284 × 10^{-6} M. Greater concentrations (2.84–284 × 10^{-6} M) were more favorable but had equal activity at the 95% confidence level. A cysteine concentration of 2,840 × 10^{-6} M decreased the reaction of the system from pH 8.1 to 3.8, and survival of cells of *E. coli* was influenced adversely.

The favorable effect of cysteine may result from reaction of the sulfhydryl group with toxic halates, as suggested by Johannesson (1957), but there are other possibilities worthy of consideration. The ability of amino acids to chelate metals is well established (Martell and Calvin, 1952; Greenstein and Winitz, 1961); cysteine may exert a favorable effect by removal of metals present in sea water at toxic levels, or by solubilizing and thereby rendering

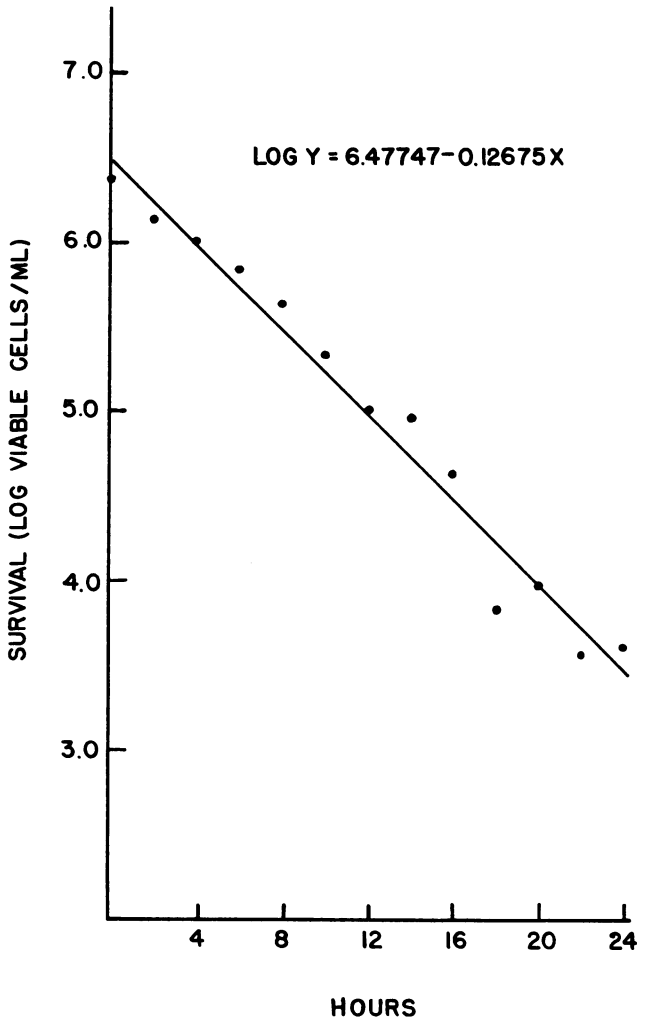

FIG. 1. *Relationship between survival of cells of Escherichia coli in artificial sea water and time.*

TABLE 1. *Influence of concentration of cysteine on survival of Escherichia coli in sea water*

Cysteine added	Survival after 24 hr
$M \times 10^{-6}$	*log %*
0	−2.456
0.284	−0.876
2.84	1.250
28.4	1.041
284.0	0.798
2,840.0	−2.301

required trace metals more available to cells. It is feasible also that cysteine acts by modifying the oxidation-reduction potential of sea water, which is poorly poised. To determine which if any of these possibilities was the more probable, the influence of various sulfhydryl and disulfide compounds on the survival of cells of *E. coli* in sea water was measured. Moreover, the effect of compounds which lack a functional sulfur group but are capable of affecting oxidation-reduction potential was determined.

Sulfhydryl compounds. Each of the following sulfhydryl compounds was tested at concentrations of 0.0284–28.4 $\times$ 10^{-6} M for its effect on survival of *E. coli* in sea water: cysteine, cysteamine, British Anti-Lewisite (BAL), glutathione, thioglycolate, and thiosulfate. The inorganic compound, sodium thiosulfate, was reported by Noble and Gullans (1955) to increase the survival time of cells of coliform organisms in lake water. Each of the six compounds is able to influence oxidation-reduction potential, chelate heavy metals, and react with halates.

The survival time of cells was shorter in water that received cysteamine than in untreated water. By applying the value of 0.622 log units as the difference between means required for significance, it was concluded that BAL and glutathione, at a concentration of 28.4 $\times$ 10^{-6} M only, increased survival of the test organism in sea water. Thioglycolate and thiosulfate had no effect. Survival of cells of *E. coli* was benefited consistently, and the effect was a function of concentration only in the case of cysteine; it appeared that the ability of this compound to increase survival of cells of *E. coli* in sea water was not due solely to the sulfhydryl group.

Disulfide compounds. The influence of the following disulfide compounds on survival of cells of *E. coli* in sea water was measured: cystamine, cystine, and hydrosulfite. Each was tested at four levels, varying from 0.0284–28.4 $\times$ 10^{-6} M. All have been reported to protect cells from the damaging effect of ionizing radiation (Kelner et al., 1955).

The results demonstrated that both cystamine and cystine significantly increased survival of cells of *E. coli* in sea water. Cystamine was effective at 0.28 and 2.84 $\times$ 10^{-6} M only, whereas cystine was beneficial throughout the concentration range tested and survival of the test organism increased with each increase in cystine level. In water containing 2.84 and 28.4 $\times$ 10^{-6} M cystine, survival of the test bacterium was approximately 4,000 times greater than in untreated water. The inorganic disulfide, sodium hydrosulfite, was ineffective at all concentrations tested.

Compounds affecting oxidation-reduction potential. Measurements were made to determine whether compounds that lacked functional sulfur groups but were capable of influencing oxidation-reduction potential had any effect on survival of cells of *E. coli* in sea water. The substances tested were alpha-tocopherol, ascorbic acid, menadione, and riboflavine. Each compound was tested at four concentrations, ranging from 0.028 to 28.4 $\times$ 10^{-6} M; in no case was a significant effect observed.

The results of the foregoing studies suggested that the ability of the cysteine molecule to increase the survival time of cells of *E. coli* in sea water involved more than the presence therein of a sulfhydryl group or the ability to influence oxidation-reduction potential. In a further effort to clarify the mode of action of cysteine, a series of studies was performed to relate molecular configuration and activity.

Compounds related structurally to cysteine. Table 2 summarizes the results of six separate experiments in which the effects of cysteine and 18 other compounds on survival of *E. coli* in artificial sea water were compared at a concentration of 28.4 $\times$ 10^{-6} M.

It is apparent that some compounds demonstrated activity that was as great as or greater than that of cysteine. This was true of serine, methionine, glutamic acid, reduced and oxidized glutathione, cystine, homocysteine,

TABLE 2. *Influence of cysteine and related compounds on survival of Escherichia coli in sea water*

Compound added	Survival after 24 hr
28.4 $\times$ 10^{-6} M	*log %*
Experiment I	
None	−1.149
Cysteine	1.444
Serine	1.312
Methionine	1.065
Na-pyruvate	−0.775
Glutamic acid monohydrate	1.004
Experiment II	
None	−1.222
Cysteine	1.391
Alanine	−0.143
Na-propionate	−2.553
Glycine	0.395
Na-acetate	−3.155
Experiment III	
None	−0.310
Cysteine	1.680
Cystamine	−1.523
Cysteamine	−2.699
Glutathione (oxidized)	1.927
Glutathione (reduced)	1.548
Experiment IV	
None	−1.155
Cysteine	0.713
Cystine	1.417
Homocysteine thiolactone	0.121
Homocystine	1.158
Serine	0.740
Experiment V	
None	−1.585
Cysteine	1.407
Cysteic acid	1.491
Taurine	−1.553
Experiment VI	
None	−2.795
Cysteine	1.182
Aspartic acid	0.990

homocystine, cysteic acid, and aspartic acid. Alanine and glycine increased the survival time of cells of the test bacterium in sea water, but the magnitude of the effect was less than that obtained with cysteine. Pyruvate and taurine had no significant effect, and the following compounds were detrimental rather than beneficial: propionate, acetate, cystamine, and cysteamine.

In general, disulfides were more active than the corresponding sulfhydryl compounds. At equal molar concentrations, cystine was more beneficial than cysteine, homocystine was more favorable than homocysteine, but oxidized and reduced glutathione had equal activity. Each of the three amino acid moieties (cysteine, glutamic acid, and glycine) of the glutathione molecule was in itself capable of exerting a favorable influence on survival of *E. coli*.

The ability of analogues of cysteine (serine, the hydroxy analogue; cysteic acid, the sulfonic acid analogue; and aspartic acid, the carboxyl analogue) to increase the survival time of cells of *E. coli* in sea water indicates that activity was not dependent solely on the presence in the molecule of a sulfhydryl group. An additional carbon atom can be added to cystine (homocystine) and cysteine (homocysteine) without loss of activity. Compounds (methionine and glutamic acid) related structurally to homocysteine had activity equal to that of cysteine.

Decarboxylation of cysteine yields cysteamine, which had no beneficial action. The disulfide cystamine was also inactive. Cysteic acid, the sulfonic acid analogue of cysteine, was equal in activity to cysteine, but taurine, the compound that results from decarboxylation of cysteic acid, was ineffective. Thus, it appears that the carboxyl group was functional and of significance. Serine demonstrated activity equal to that of cysteine, but reduction of the hydroxy group (alanine) resulted in a marked decrease but not complete loss of activity.

Deamination of alanine yields propionate or pyruvate, and deamination of glycine yields acetate. These organic acids were completely lacking in activity, whereas the amino acids significantly increase survival of the test organism in sea water. It is of interest that thioglycolate, which possesses a sulfhydryl and carboxyl group but lacks an amino group, was found to have little or no beneficial effect.

From the foregoing studies, it appears that disulfide compounds are generally more active than their sulfhydryl counterparts, but a sulfur-containing functional group is not indispensible. Moreover, the ability of a compound to increase the survival time of cells of *E. coli* in sea water does not depend on the ease with which the molecule is oxidized or reduced. It is evident that substances which do not possess both an amino and carboxyl group have little or no activity. Both substituents were present in every compound that exerted a consistent and significant beneficial effect. These observations are not consistent with the suggestion of Johannesson (1957) that cysteine renders sea water more favorable for survival of *E. coli* by reaction of the sulfhydryl group of the amino acid with iodate, but they are consistent with the possibility that cysteine acts to favor survival of cells of *E. coli* in sea water by a chelation mechanism.

The ability of amino acids to chelate metals is well established; the groups described by Martell and Calvin (1952) and Greenstein and Winitz (1961) as most functional in the reaction include R—NH_2 (primary amino), R—OH (hydroxyl), R—SH (sulfhydryl), R—COOH (carboxyl), and R—$\overset{\overset{O}{\|}}{\underset{\underset{O}{\|}}{S}}$—OH (sulfonic). These are the very substituents present in the compounds capable of increasing the survival time of cells of *E. coli* in sea water. Serine had activity similar to that of cysteine, indicating the sulfhydryl group of the latter compound was replaceable by a hydroxyl group. Moreover, the beneficial effects of cysteic and aspartic acid demonstrated that it is possible to substitute a sulfonic or carboxyl for the sulfhydryl group of cysteine without significant loss of activity. In the case of alanine, which differs from cysteine in that it contains a hydrogen atom in place of the sulfhydryl group, activity was decreased greatly from that of compounds having substituents more effective as electron donors in chelation reactions. On the basis of the foregoing studies, it is suggested that cysteine acts to increase survival of cells of *E. coli* in sea water by virtue of its ability to chelate metal ions. However, further study is required before this possibility is established as fact.

Acknowledgments

The authors wish to express their appreciation to C. L. Grant, Engineering Experiment Station, University of New Hampshire, who assisted in the design of experiments and performed the statistical analyses.

This investigation was supported in part by research grant E1437 from the National Institute of Allergy and Infectious Diseases, U.S. Public Health Service.

LITERATURE CITED

Carlucci, A. F., and D. Pramer. 1960*a*. An evaluation of factors affecting the survival of *Escherichia coli* in sea water. I. Experimental procedures. Appl. Microbiol. **8**:243–247.

Carlucci, A. F., and D. Pramer. 1960*b*. An evaluation of factors affecting the survival of *Escherichia coli* in sea water. II. Salinity, pH, and nutrients. Appl. Microbiol. **8**:247–250.

Carlucci, A. F., and D. Pramer. 1960*c*. An evaluation of factors affecting the survival of *Escherichia coli* in sea water. III. Antibiotics. Appl. Microbiol. **8**:251–254.

Carlucci, A. F., and D. Pramer. 1960*d*. An evaluation of factors affecting the survival of *Escherichia coli* in sea water. IV. Bacteriophages. Appl. Microbiol. **8**:254–256.

Carlucci, A. F., P. V. Scarpino, and D. Pramer. 1961. Evaluation of factors affecting the survival of *Escherichia coli* in sea water. V. Studies with heat- and filter-sterilized sea water. Appl. Microbiol. **9**:400–404.

DIXON, W. J., AND F. J. MASSEY. 1957. Introduction to statistical analysis. McGraw-Hill Book Co., New York.

GREENSTEIN, J. P., AND D. M. WINITZ. 1961. Chemistry of the amino acids, vol. 1. John Wiley & Sons, Inc., New York.

JOHANNESSON, J. K. 1957. Nature of the bactericidal agent in sea water. Nature **180**:285–286.

JOHANNESSON, J. K. 1958. Oxidized iodine in sea water. Nature **182**:251.

KELNER, A., W. D. BELLAMY, G. E. STAPLETON, AND M. R. ZELLE. 1955. Symposium on radiation effects on cells and bacteria. Bacteriol. Rev. **19**:22–44.

MACLEOD, R. A., AND E. ONOFREY. 1956. Nutrition and metabolism of marine bacteria. II. Observations on the relation of sea water to the growth of marine bacteria. J. Bacteriol. **71**:661–667.

MARTELL, A. E., AND M. CALVIN. 1952. Chemistry of the metal chelate compounds. Prentice-Hall, Inc., New York.

NOBLE, R. E., AND O. GULLANS. 1955. Influence of sodium thiosulfate on the survival of coliform organisms in stored samples of untreated lake water. J. Bacteriol. **70**:249–250.

SHAW, T. I., AND L. H. N. COOPER. 1957. State of iodine in sea water. Nature **180**:250.

SUGAWARA, K., AND K. TERADA. 1958. Oxidized iodine in sea water. Nature **182**:250–251.