JOURNAL OF BACTERIOLOGY, Oct., 1965
Vol. 90, No. 4

Inhibitory Action of Selenite on *Escherichia coli*, *Proteus vulgaris*, and *Salmonella thompson*[1]

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Received for publication 23 April 1965

ABSTRACT

WEISS, K. F. (Iowa State University, Ames), J. C. AYRES, AND A. A. KRAFT. Inhibitory action of selenite on *Escherichia coli*, *Proteus vulgaris*, and *Salmonella thompson*. J. Bacteriol. **90**:857–862. 1965.—The resistance of three microorganisms, *Escherichia coli* (ISU-41), *Proteus vulgaris* (ISU-37c), and *Salmonella thompson* (ISU-86-2), to increasing concentrations of selenite was determined. *E. coli* was completely inhibited by 1.25% sodium hydrogen selenite, and 0.25% sodium hydrogen selenite caused a pronounced lag. *P. vulgaris* survived selenite concentrations of over 3%. *S. thompson* was inhibited completely by 3% selenite but not by 2.5%, although there was a considerable lag and a decrease in total growth. The relationship of growth, uptake, and reduction of selenite was determined. The susceptible *E. coli* incorporated up to twice as much selenium as did the other two organisms during the early stages of incubation. Radioautographs of seleno analogues of sulfur-containing amino acids revealed the presence of seleno-cystine in all three organisms, and seleno-methionine in *E. coli*. Compounds having R_F values corresponding to possible oxidation products of seleno-methionine were present in the hydrolysates of *P. vulgaris* and *S. thompson*. Kinetic aspects of selenite uptake, rather than the ultimate localization of selenite in the cell protein, appear to be the factors that determine the degree of resistance or of susceptibility to selenite.

The inhibitory action of selenite on susceptible microorganisms in culture media manifests itself in retarded growth and is accompanied by reduction of selenite to metallic selenium. It is believed that, in part, the toxicity of selenite for microorganisms may be attributable to the incorporation of seleno analogues of sulfur-containing amino acids into proteins (Tuve, Ph.D. Thesis, Cornell University, Ithaca, N.Y., 1958). Seleno-methionine, seleno-cystine, and several unidentified intermediates have been isolated from seleniferous protein hydrolysates of *Escherichia coli*.

Evidence exists for a nonmetabolic reaction by which inorganic selenium compounds may exert their toxic effect upon microorganisms (Scala and Williams, 1962).

The purpose of the present study was to determine whether the amount of selenium incorporated into cell protein, or the rate of uptake, was important in the inhibitory action of selenite on bacteria.

[1] Journal Paper J-4966 of the Iowa Agricultural and Home Economics Experiment Station, Ames, Iowa. Project 1392, Center for Agricultural and Economic Development cooperating.

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MATERIALS AND METHODS

Laboratory strains of *E. coli* (ISU-41), *Proteus vulgaris* (ISU-37c), and *Salmonella thompson* (ISU-86-2) provided by the Department of Bacteriology, Iowa State University, were maintained as stock cultures on nutrient agar slants.

The basic Tryptone-phosphate medium for determining selenite resistance of the organisms consisted of tryptone (5.0 g per liter), K_2HPO_4 (7.5 g per liter), and KH_2PO_4 (2.5 g per liter) at *p*H 7.0 ± 0.1. Plate counts were made at 0, 12, 24, and 48 hr; nutrient agar was the plating medium.

Total selenite uptake was determined as follows: Samples (5 ml) of the cultures containing radioactive selenite were withdrawn at appropriate intervals during growth and filtered through Millipore (porosity, 0.22 μ) filters (Millipore Filter Corp., Bedford, Mass.). The cells were washed four times with 10-ml volumes of distilled water to remove adhering soluble radioactive material and to wash out the unbound radioactive compounds. The filters were dried and placed on aluminum planchets, and the radioactivity was determined with a Picker proportional flow counter (Picker X-Ray Corp., White Plains, N.Y.) equipped with an ultrathin window (less than 0.15 mg/cm^2).

Net uptake of selenite was taken as the numerical difference between total uptake of selenite, as determined by the radioactive measurements, and the reduction of selenite to metallic selenium

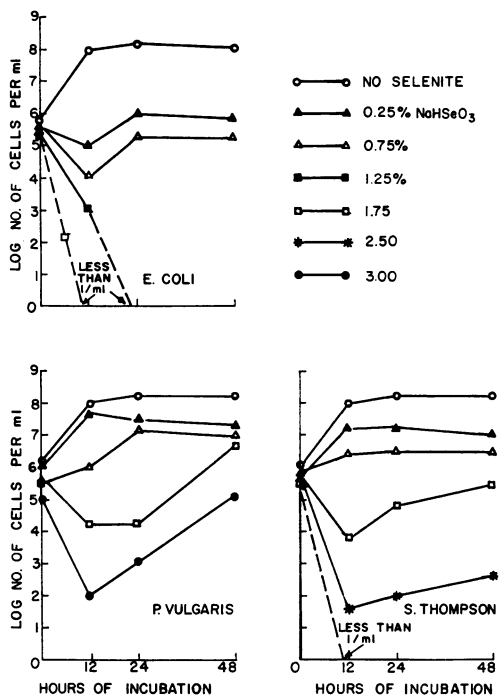

FIG. 1. *Effect of selenite concentration on growth of Escherichia coli, Proteus vulgaris, and Salmonella thompson.*

estimated from the standard graph by the method of Falcone and Nickerson (1960).

The procedures suggested by Roberts et al. (1955) were generally followed for routine fractionation of cells.

A selenite ($NaHSeO_3$) concentration of 0.1% containing Se^{75} was used to give a specific activity of 10^4 counts per min per ml in a total volume of 1 liter. The inoculated broth was incubated at 37 C for 48 hr on a rotary shaker. After centrifugation, the cells were washed with physiological saline until the supernatant fluid was free from radioactivity. Enzymatic hydrolysis only was employed because of the labile nature of organo-selenium compounds. The hydrolytic procedure utilized the enzymes pepsin, erepsin, and pancreatin (Tuve, Ph.D. Thesis, 1958).

Ion-exchange columns were prepared from the hydrogen-saturated cation exchange resin, Dowex 50 × 8. The resin was prepared by treating Dowex 50 × 8, 200 to 400 mesh, with 6 N hydrochloric acid until the supernatant liquid was colorless. The resin was washed with distilled water to approximately *p*H 6 and poured as a slurry into 10-ml burettes. After the hydrolysate was placed on the Dowex 50 column, the resin was washed with 15 ml of water, and the column was eluted successively with 5-ml portions of 1.5 and 6 N hydrochloric acid. The fractions containing the highest radioactivity were concentrated by drying at 50 C to remove the excess hydrochloric acid. The resulting crystals of amino acid and peptide hydrochlorides from the different fractions were combined by dissolving in water, and a sample was withdrawn for investigation by paper chromatography and radioautography.

Descending one-dimensional chromatography was employed throughout with Whatman no. 1 paper (10 × 45 cm). The solvent mixture used was isopropyl alcohol–formic acid–water, 70:10:20 (Roberts et al., 1955). The solvent front was allowed to travel to approximately 3 cm from the bottom of the paper; the chromatograms were then dried at 45 C for 30 min, and radioautographs were prepared. Samples of seleno-methionine, seleno-cystine, seleno-cystathionine, and selenite were included with each set of chromatograms containing the unknown compounds. Chromatograms containing the known amino acids were developed in a 0.25% (w/v) glacial acetic acid, and were finally heated for 15 min at 45 C (Mizell and Simpson, 1961). The presence of selenite was determined by dipping the chromatogram in acetone containing ascorbic acid so that the compound was reduced to the red elemental selenium.

Ninhydrin was also used for positive identification of seleno-amino acids by the "fingerprint" method (Tuve and Williams, 1957).

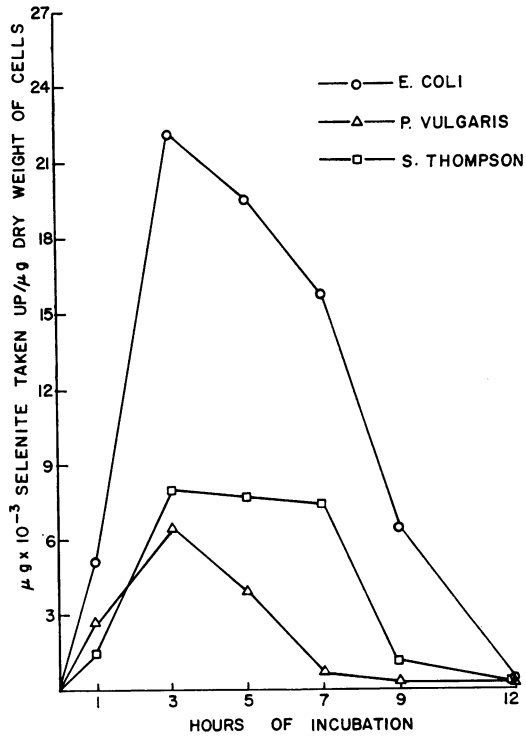

FIG. 2. *Net uptake of selenite by Escherichia coli, Proteus vulgaris, and Salmonella thompson at a selenite concentration of 50 μg/ml in Tryptone-phosphate broth.*

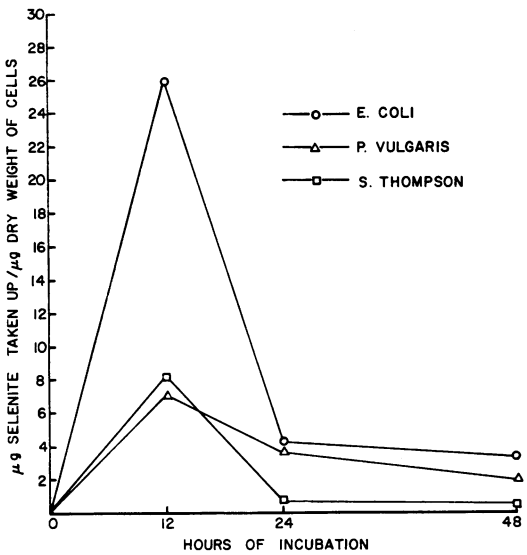

FIG. 3. *Net uptake of selenite by Escherichia coli, Proteus vulgaris, and Salmonella thompson at a selenite concentration of 7,500 μg/ml in Tryptone-phosphate broth.*

The dried radioactive paper chromatograms were placed in air-tight cardboard folders or in No-Screen steel casettes, and Kodak No-Screen X-ray films (Eastman Kodak Co., Rochester, N.Y.) were exposed to the radioactive material on the chromatograms. After exposure, the films were developed in Kodak D-19 and treated in acid fixer (hypo) until clear. The developed films were washed in running water and dried.

RESULTS

The different degrees of resistance to increasing concentrations of sodium selenite exhibited by the three bacterial species are demonstrated in Fig. 1. A concentration of 1.25% $NaHSeO_3$ resulted in complete inhibition of *E. coli*, and at a concentration as low as 0.25% sodium selenite there was a pronounced lag; the number of viable cells at the end of 48 hr was about the same as the inoculum.

P. vulgaris survived selenite concentrations of 3%, although there was a sharp decrease in viable organisms during the first 12 hr. At the lowest concentration, 0.25%, there was no inhibition during the initial 12 hr; thereafter, the number of viable cells decreased.

S. thompson was completely inhibited by 3% sodium selenite; 2.5% caused a considerable lag and decrease in total growth. Concentrations of less than 1% had about the same effect as on *P. vulgaris*.

Studies of selenite utilization by the three organisms were conducted with two widely different selenite concentrations (50 and 7,500 μg/ml) to determine whether differences in the kinetics of selenite uptake could account for the great variations in selenite tolerance observed.

It is of interest to note that, for both selenite concentrations, the nontoxic (50 μg/ml) as well as the selectively inhibitory (7,500 μg/ml), the same general pattern of net uptake of selenite was obtained (Fig. 2 and 3). At the low selenite concentration, there was a massive uptake of selenium by *E. coli* within the first 3 hr of incubation (Fig. 2). The uptake by the other two organisms during the same time was less than half of that of *E. coli*. A similar pattern was exhibited at the high selenite concentration (Fig. 3). The susceptible *E. coli*, which sustained a considerable lag at this concentration (see Fig. 1) during the first 12 hr, took up 26 μg of selenium per μg (dry weight) of cells within this time interval. The resistant *P. vulgaris* and *S. thompson* had a net uptake of only 7.0 and 8.2 μg of selenium, respectively, per μg (dry weight) of cells.

The relationship of total utilization, reduction, and net uptake of selenite by the three microor-

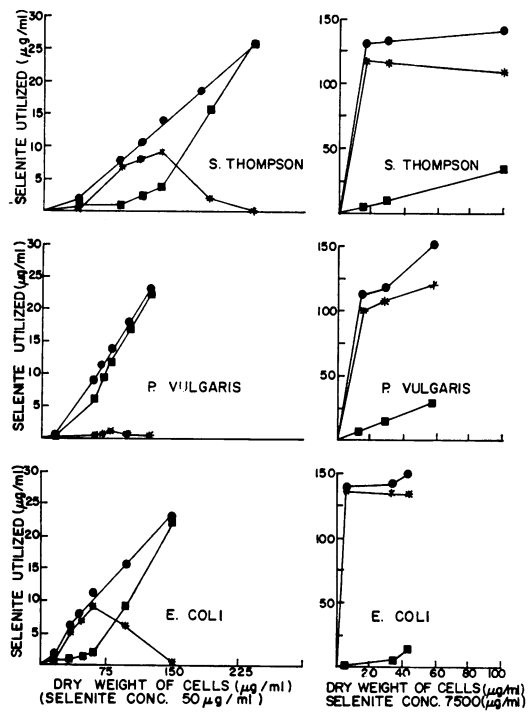

FIG. 4. *Selenite utilization by Escherichia coli, Proteus vulgaris, and Salmonella thompson. Measurements made for the low selenite concentrations at 1, 3, 5, 7, 9, and 12 hr of incubation, and at 12, 24, and 48 hr of incubation for the high selenite concentration. Symbols:* ●, *total uptake;* ■, *reduction;* *, *net uptake.*

TABLE 1. *Distribution of selenium in cell fractions*

Fraction	Percentage range of radioactivity*		
	Escherichia coli	*Proteus vulgaris*	*Salmonella thompson*
Washed cells	100	100	100
Cold trichloroacetic acid-soluble	0.029–0.080 (0.055)	0.026–0.050 (0.036)	0.153–0.190 (0.168)
Alcohol-soluble	0.290–0.580 (0.410)	0.075–0.175 (0.118)	0.224–0.300 (0.251)
Alcohol ether-soluble	0.350–0.600 (0.453)	0.260–0.364 (0.325)	0.750–0.833 (0.734)
Hot trichloroacetic acid-soluble	0.023–0.050 (0.034)	0.015–0.027 (0.021)	0.029–0.060 (0.046)
Residual protein	88.231–91.487 (89.902)	86.419–90.747 (88.755)	93.145–96.015 (94.395)
Total recovered	88.923–92.797 (90.854)	86.795–91.363 (89.255)	94.301–97.398 (95.594)

* Values in parentheses are the mean of three determinations.

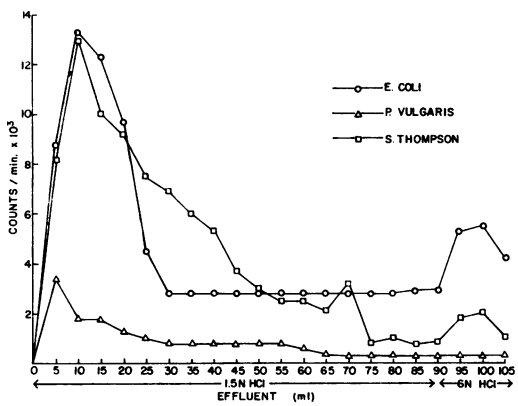

FIG. 5. *Column chromatography of protein hydrolysates of Escherichia coli, Proteus vulgaris, and Salmonella thompson.*

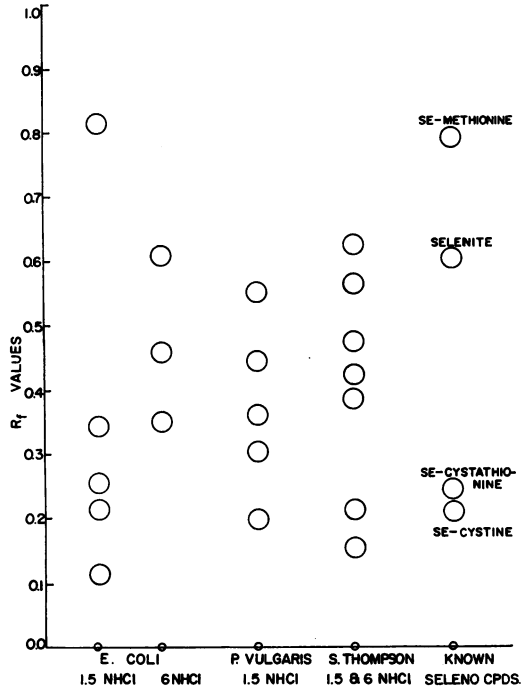

FIG. 6. *Line drawing of radioautographs and chromatograms of HCl effluents of Escherichia coli, Proteus vulgaris, and Salmonella thompson.*

ganisms is demonstrated more clearly in Fig. 4. At the low selenite concentration, utilization of selenite was linear with increasing dry weight of cells after 1 hr of incubation for *S. thompson* and *P. vulgaris*. With *E. coli*, after a rapid uptake of selenium between the 1st and 3rd hr of incubation, total selenite uptake also became linear. After 7 hr, net uptake of selenite decreased sharply while the rate of reduction increased. This was true for all three organisms, although the uptake and reduction rates varied considerably.

In the presence of high concentrations of selenite when total growth was retarded, total and net uptake were approximately the same, especially for *E. coli*, which showed very little reduction for the first 24 hr. Total utilization and net uptake were again more than twice as high for *E. coli* as for the other two species during the first 12 hr. This rapid and extensive uptake of selenium in the early stages of growth by *E. coli* may, at least in part, have accounted for the considerable lag exhibited by this organism.

Inasmuch as variable amounts of $S^{35}O_4^{-2}$ have been found in the nonprotein fraction of *E. coli* (Roberts et al., 1955), the distribution of Se^{75} in those cell fractions of *E. coli*, *P. vulgaris*, and *S. thompson* was determined (Table 1).

A typical distribution of $S^{35}O_4^{-2}$ in the five fractions of *E. coli* as given by Roberts et al. was as follows: cold trichloroacetic acid-soluble, 23.6%; alcohol-soluble, 13.7%; alcohol ether-soluble, 0.54%; hot trichloroacetic acid-soluble, 2.05%; residual protein, 56.1%.

The distribution of Se^{75} is given here for comparative purposes only, however, and no correla-

tion to the distribution of S^{35} is sought. In the present work, there were no marked differences in the distribution of Se^{75} among the fractions of the three bacterial species (Table 1).

Selenium-containing amino acids from the residual proteins of *E. coli*, *P. vulgaris*, and *S. thompson* were eluted from a Dowex 50 (H^+ form) cation-exchange column with 90 ml of 1.5 N hydrochloric acid in 5-ml portions, followed by 30 ml of 6 N hydrochloric acid also in 5-ml fractions. The elution patterns are presented in Fig. 5. *E. coli* showed a rather sharply defined peak between 5 and 25 ml of 1.5 N HCl and a smaller peak after the addition of 6 N HCl. From *P. vulgaris*, only a few fairly active fractions were obtained with 1.5 N HCl. The pattern for *S. thompson* was similar to that obtained for *E. coli*, except for a more gradual decrease in activity after the first peak, and a very low second peak.

The most active 1.5 N HCl and 6 N HCl fractions were dried at 50 C to remove hydrochloric acid, were suspended in water, and were dried again. The resulting crystals were combined by dissolving in water. The fractions containing 1.5 N HCl and 6 N HCl from *E. coli* only were used separately. The activities of the 6 N HCl fractions from *P. vulgaris* and *S. thompson* were very low; hence, these were combined with the 1.5 N HCl fractions for the respective organisms.

The four final solutions were chromatographed, and the chromatograms were radioautographed to locate the position of the radioactive material. Figure 6 is a line drawing of the chromatograms and radioautographs obtained from the three microorganisms. For all three organisms, there were spots with R_F values corresponding to synthetic seleno-cystine. As concluded from the elution pattern, *S. thompson* gave the largest number of radioactive spots, but neither seleno-cystathionine nor seleno-methionine could be detected. The spot with an R_F of 0.62 was "fingerprinted" with carrier selenite, and its location was determined by dipping the irrigated and dried chromatogram in an ascorbic acid-acetone solution. A distinct red spot appeared on the paper which superimposed exactly with the radioactive spot on the radioautograph, thus proving the presence of selenite in the original fraction. The other spots were not further investigated.

P. vulgaris yielded only five spots. Values for seleno-cystine superimposed, but neither seleno-cystathionine nor seleno-methionine could be detected. The last spot (R_F = 0.55) was very weak, and no attempt was made to "fingerprint" it, although it could have been selenite or an unknown organic seleno-compound. Tuve (Ph.D. Thesis, 1958), in his studies on *E. coli*, obtained a compound having an R_F value of 0.55 in the same solvent.

The 1.5 N HCl fractions of *E. coli* revealed seleno-cystine, seleno-cystathionine, and seleno-methionine, and proof was obtained by "fingerprinting." No seleno-cystine was found in the 6 N HCl fractions, but the presence of selenite was proved, suggesting that the selenite identified for *S. thompson* was present in the 6 N HCl fractions from this organism.

DISCUSSION

The growth curves served to illustrate the effect of sodium selenite on the three organisms in a liquid enrichment medium. The pronounced lag (Phoenix phenomenon) in the earlier stages of growth of *E. coli*, even at relatively low selenite concentrations, is typical also for many other organisms, and is the basis for the use of the selenite enrichment media (Leifson, 1936). The strain of *P. vulgaris* used was very resistant to selenite, and the organism was a valuable test specimen, even though such high selenite resistance is not typical for the entire genus.

The fact that the weight of selenium taken up by all three bacterial species by far exceeded the dry weight of the cells indicated that much of the selenite was adsorbed by the cells in addition to being metabolized in the synthesis of seleno-analogues of sulfur-containing amino acids. Tuve and Williams (1961) ruled out the possibility that incorporation of selenite was due to adaptation, adsorption, or deposition of elemental selenium. These phenomena can hold true only for very low selenite concentrations, as the results obtained here indicate; furthermore, it was shown by Falcone and Nickerson (1960) that selenium can become chemically bound to thiol groups of proteins.

At least two different mechanisms of selenite toxicity were suggested by Tuve and Williams (1961): (i) the reaction of selenite with sulfhydryl groups, and (ii) the incorporation of selenium into analogues of sulfur compounds. The formation of seleno-amino acids was determined, and from Fig. 6 it may be observed that, since several of the unidentified compounds from the three organisms have similar R_F values, similarity in the metabolic pattern is suggested. The absence of seleno-methionine in *P. vulgaris* and *S. thompson* was rather surprising, since an accumulation of this innocuous seleno-amino acid at the expense of the toxic seleno-cystine could have accounted for the resistance to selenite exhibited by these two species. We have assumed that some of the unidentified spots represent oxidation products of seleno-methionine, since their R_F values are similar to those obtained by Tuve (Ph.D. Thesis, 1958). These spots were present on the radioautographs of *E. coli*, indicating that conversion

per se of cystine to methionine is not a differentiating factor in selenite resistance among the three organisms. The kinetic aspect of selenite uptake, rather than the ultimate qualitative localization of selenite in the cell protein, seems to be the factor that determines the degree of resistance or sensitivity to selenite.

During the early stages of incubation, a sharply increased uptake of selenium coincided with high susceptibility to selenite (Fig. 1 to 4). It is possible that the relative ratios of seleno-amino acids to their sulfur analogues cause the cell protein to become partially or completely inactive, thereby resulting in less total growth, a reduced growth rate, or complete cessation of growth.

The toxicity due to reaction of selenite with sulfhydryl groups should not be overlooked. In different organisms, quantitative differences in loss of functional SH^- groups because of oxidation may result in increased resistance or sensitivity to a given concentration of selenite.

ACKNOWLEDGMENT

This investigation was supported by Public Health Service research grant EF-00113-10 from the Division of Environmental Engineering and Food Protection.

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