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The inhibitory action of selenite on Escherichia coli, Proteus vulgaris and Salmonella thompson

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THE INHIBITORY ACTION OF SELENITE ON *ESCHERICHIA COLI*, *PROTEUS VULGARIS* AND *SALMONELLA THOMPSON*

by

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TABLE OF CONTENTS

I. INTRODUCTION

Practical use of the selectively inhibitory action of selenium salts on microorganisms, as a diagnostic tool, was not tried until 1936 when several selenite enrichment media were developed. Originally, these media were devised to isolate Salmonella typhosa and Salmonella paratyphi from feces, sewage, and possibly fluid milk.

With the appearance of dried egg on the consumer market and an almost simultaneous increase in outbreaks of salmonellosis, public health authorities and food scientists began to search for suitable media and procedures to detect and isolate food-borne Salmonellae. The employment of enrichment media was necessary, since in eggs and egg products these organisms may be greatly outnumbered by other contaminants such as coliforms, Proteus and pseudomonads. Tetrathionate and sodium selenite formulations were adopted for routine examination of eggs and other food products.

Selenite enrichment broth became the medium of choice for many agencies concerned with the isolation of Salmonellae from foods. The inhibitory action of selenite on susceptible microorganisms in culture media manifested itself in retarded growth and was accompanied by a greater or lesser degree of

reduction of selenite to metallic selenium. It was recognized that the toxicity of selenite to a given organism was not only a function of the concentration of the selenium salts, but depended also on factors such as pH, total salt content, and abundance of proteinaceous matter of the medium.

The nature of the toxic action of selenite has been the subject of much study. Early work was done on plants causing "alkali disease" in animals. Such plants were found to contain high levels of selenium. It was discovered that selenium in toxic grains was associated with the protein of the plants, and it was postulated that selenium might partially replace sulfur in sulfur-containing amino acids of the toxic proteins. It is now believed that toxicity of selenite for microorganisms may at least partially be due to the incorporation of selenoanalogues of sulfur-containing amino acids into proteins. These "false proteins" are biochemically altered in such a manner as to render them toxic. Selenomethionine, seleno-cystine, and several unidentified intermediates have been isolated from seleniferous protein hydrolysates of E. coli.

There is also evidence in the literature for a non-metabolic reaction by which inorganic selenium compounds may

exert their toxic effect upon microorganisms. Selenite may oxidize the sulfhydryl groups of biologically essential compounds (cysteine in glutathione and proteins, and coenzyme A), destroying their biological properties.

This thesis compares the effect of selenite on three different microorganisms under various conditions. The purpose of the study is to elucidate possible differences in mechanisms by which selenite exerts its selectively inhibitory effect on different bacterial species.

II. LITERATURE REVIEW

A. Introduction

The use of selenite as a bacteriostatic agent in a selective medium for the isolation of typhoid bacilli (Salmonellae) was first suggested by Guth (1916). Earlier investigators had already noted that the growth of most coliform organisms was inhibited to a greater degree than that of Salmonellae by selenium salts. Guth found that the inhibitory action of sodium selenite against coliform bacteria was greatly dependent on the reaction of the medium. Inhibition of growth decreased with increasing alkalinity; the more alkaline the medium, the more selenite was required. He also noted that susceptibility to selenite varied considerably among the different coliforms and Salmonellae; however, differences between fresh and old isolates could not be observed.

Levine (1913) studied the toxic effect of selenium compounds upon yeasts, higher plants and animals. He used selenium dioxide (selenious acid), selenic acid, sodium selenite, sodium selenate, and potassium selenocyanide. Levine arranged the compounds in the following series of

diminishing toxic effects: selenium dioxide (selenious acid), selenic acid, sodium selenite, sodium selenate, potassium selenocyanide. This order agreed well with that found later by Levine (1925) in a study of selenium compounds upon the growth of bacteria with a view toward a possible relation between growth and reduction.

Reduction of the selenium salts took place only where growth occurred and the profuseness of reduction parallelled that of growth. Selenic acid was reduced more slowly than selenious acid, whereas sodium selenate was not reduced to free selenium. Levine stated that reduction was intimately associated with vigor of growth, and was caused by the bacterial cells. Too high a selenium ion concentration inhibited growth and gave diminished reduction; heating the cultures to above 60°C destroyed the reducing power. The "reductase" elaborated by the bacterial cells was thought to be an endo-enzyme reducing energetically in the presence of an activating substance or co-enzyme which was capable of being dissolved from the cell or of being removed by Berkefeld filtration.

Another reaction of selenite and selenious acid shown by microorganisms is alkylation to volatile compounds of

characteristic odor. Hofmeister (1894) found that selenite was alkylated to methyl selenide in the tissues of higher animals. Maassen (1904), in contrast, stated that the end product of alkylation of selenite by microorganisms was ethyl selenide. Gosio (1905) pointed out that reduction and alkylation (ethyl selenide synthesis) were related but not in a manner such that with increased reduction there was increased alkylation; on the contrary, reduction could completely suppress the formation of volatile seleno-compounds.

B. Selenite Enrichment Media

On the basis of the above-mentioned findings, Leifson (1936a) developed several selenite enrichment media. He proposed the use of selenite F for the isolation of typhoid and paratyphoid bacilli (S. typhosa and S. paratyphi) from feces, selenite M for isolation from milk, and selenite S for isolation from sewage. Leifson pointed out that, as with most substances, the toxicity of sodium selenite for bacteria depended to a large extent upon the basic medium, and various degrees of toxicity could be obtained by changing the composition of the basic medium. The degree of toxicity of sodium selenite was influenced in the following manner: (1) toxicity

decreased with increasing pH (as shown by Guth, 1916), and was many times greater at pH 6.5 than at pH 8.0. (2) sodium chloride decreased toxicity for most bacteria. In a simple peptone medium the selenite salt was much more toxic than in the presence of 0.5 to 1% sodium chloride. (3) phosphates had a striking effect in reducing the toxicity of selenites. (4) salts such as sulfite and nitrite rendered a selenite medium extremely toxic. (5) beef extract and especially meat infusion reduced the toxicity of selenite. According to Leifson, the action of selenite enrichment media was such that the number of viable coliform and Proteus organisms decreased during the first 8 to 12 hours in the medium, followed by a more or less rapid increase. The Salmonellae, in contrast, multiplied fairly rapidly from the start and soon greatly outnumbered the colon and Proteus bacilli, assuming that equal numbers were present initially. Pyocyaneus (Pseudomonas aeruginosa), however, showed only a brief lag, the enterococci reached a rather low plateau, but Shigella flexneri and the Alcaligenes species were completely inhibited.

Leifson (1936b) briefly summarized his findings and conclusions up to that time regarding the use of sodium selenite

as a differential bacterial growth inhibitor. Salmonellae, Vibrio comma and P. aeruginosa could grow in infusion media at pH 7.0 containing as much as 1.0% sodium selenite. Most other bacteria were much more sensitive. The coliforms were considerably inhibited with 0.2%, whereas the Shigellae would not grow with 0.1% sodium selenite.

A study of the use of sodium selenite as a selective bacteriostatic agent was conducted by Gohar (1943). He employed solid as well as liquid selenite media and found that instead of total inhibition, only a delay of growth occurred because of a prolonged lag phase. He also observed that organisms sensitive to selenite could grow on solid selenite media in the neighborhood of bacteria resistant to selenite apparently because of the utilization of the selenium salt by the latter. This, too, was noted when fluid media were used. Thus, when peptone water containing sodium selenite was inoculated with a mixture of selenite-resistant and selenite-sensitive organisms, the former grew first causing a heavy deposit of a red sediment probably consisting of selenium. After a few days of incubation, the sensitive organisms also showed evidence of multiplication because of the utilization of some selenite by the resistant cells.

Gohar pointed out that inhibition was almost entirely due to bacteriostatic action of selenite, but that the selenium salt had very little or no bactericidal power.

The selenite F enrichment medium introduced by Leifson (1936) originally was intended only for the isolation of Salmonellae from fecal matter. The S and M formulations were designed for liquid specimens leaving selenite F as the selenite-containing enrichment medium of choice for the examination of solid material other than feces. Routine examination in the U.S.A. of any solid food stuff for the presence of Salmonellae is, however, of a rather recent date, although Salmonella species have long been recognized as potential food poisoning organisms (Savage, 1932; Bornstein, 1943).

A major stimulus for the detection of Salmonella in foods was provided by reports from The British Food Mission beginning early in 1942, indicating that Salmonellae were being isolated from dried-egg powder received in the British Isles for food purposes. The British authorities emphasized the undesirability of such contamination in a food product, and U.S. and Canadian investigators began intensive studies of the Salmonella content of eggs and egg products (Seligmann

et al., 1943; Gibbons and Moore, 1944a, 1944b; Schneider, 1946; Solowey et al., 1946, 1947; Solowey and Calesnik, 1948; Ayres and Slosberg, 1949; Ayres, 1949, 1953; Forsythe et al., 1953).

Leifson's selenite F broth, and Muller's (1923) tetrathionate broth (modified by Kauffmann 1931, 1936) came into general use in industrial laboratories as enrichment media for the isolation of Salmonella from egg products. It soon became evident, however, that the original formula of selenite F*, which gave very good results with fecal material, was not always satisfactory when used for albumen and whole egg. Consequently, attempts were made to improve the selectivity and efficiency of the existing selenite enrichment medium.

North and Bartram (1953) evaluated selenite broths of different composition. They noted that with certain peptones a better recovery of Salmonellae was obtained when the phosphate concentration was lowered from 1.0 to 0.25% if simultaneously up to 0.4 μg/ml cystine was added. Further

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*Composition of Leifson's selenite F enrichment medium.

	%
Sodium hydrogen selenite (anhydrous)	0.4
Sodium phosphates (anhydrous)	1.0
Peptone	0.5
Lactose	0.4

pH 7.0 ± .1

addition of cystine tended to produce better recovery at the 1.0% phosphate level.

The effect of the nutrient material (egg) on the selectivity and/or toxicity of selenite enrichment broth should be noted. Hurley and Ayres (1953) compared six enrichment media and investigated the effect of added egg on the performance of those media. These workers drew attention to the fact that the addition of egg, with its proteinaceous matter and antibacterial agents (lysozyme, avidin, conalbumin, and ovomucoid) instead of the stool specimens, usually examined by health authorities, resulted in a considerable change in the enrichment substrate. Albumen or whole egg either decreased bacterial growth (including that of the Salmonellae), or the selectivity of the enrichment media was reduced.

Stokes and Osborne (1955), suggested a selenite-brilliant green medium* which supported luxuriant growth of

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*Formula for selenite-brilliant green medium.

	%
Peptone	0.5
Yeast extract	0.5
Mannitol	0.5
Sodium selenite	0.4
Sodium taurocholate	0.1
Brilliant green	0.0005
Phosphate buffer pH 7.0	0.025 molar
Distilled water	

Salmonellae from very small inocula, but in marked contrast to selenite F medium, effectively inhibited strains of Proteus and Escherichia, instead of only delaying their growth for a few hours. The amount of phosphate in the medium was found to be of considerable importance. All the test organisms failed to grow when phosphate was omitted. It appeared to the investigators that inhibitory compounds (selenite and brilliant green) increased the phosphate required above the normal requirement level which might have been supplied by the yeast extract and the peptone of the medium. A relatively high phosphate concentration (0.1M) stimulated the growth of Proteus and partially inhibited some strains of Salmonella.

The selenite-brilliant green medium was modified by Osborne and Stokes (1955), who added sulfapyridine to the medium to restore selective properties which were reduced considerably by the presence of nutrient material.

Taylor et al. (1958), upon comparing several enrichment media, found that substitution of mannitol or dulcitol for lactose in the selenite F medium offered no increase in the numbers of Salmonellae isolated from naturally contaminated dried albumen. Furthermore, selenite brilliant green sulfapyridine enrichment broth did not produce more isolations of

Salmonellae, and, in some cases, was inferior to selenite F.

Silliker and Taylor (1958) re-investigated the effect of added nutrient material on the performance of liquid enrichment media. The findings were that cystine-selenite F, as well as tetrathionate broth, were adversely affected by the addition of many different kinds of food material (albumen, whole egg, yolk, gelatin, and dried beef). Gelatin and albumen caused the most severe reduction in numbers of Salmonellae recovered; egg yolk caused the least reduction. The authors suggested separation of the bacteria from the soluble food material by centrifugation as an effective means of restoring the function of enrichment media.

In a comparative study of enrichment methods, Taylor and Silliker (1961) found that pre-enrichment in non-selective media and centrifugation increased the recovery of Salmonellae considerably over the simple enrichment method.

Taylor (1961) re-examined the possible methods by which Salmonellae may be isolated and enumerated from dried albumen. Three methods were considered: (1) direct inoculation of enrichment media, (2) centrifugation of samples, (3) pre-enrichment in non-inhibitory media. The method of choice suggested was pre-enrichment; however, no comparison was made

with the method described by Sugiyama et al. (1960), who employed pre-enrichment with lauryl tryptose broth containing Salmonella anti-serum followed by "minimal" centrifugation to sediment only the flocculated organisms.

C. The Nature of Selenite Action on Microorganisms

1. Sulfur-selenium antagonism

The possibility exists that sulfur and selenium are competitive metabolic antagonists because of their close chemical similarity.

Early work on sulfur-selenium antagonism in plants was done by Hurd-Karrer (1934, 1937a, 1937b, 1938). She observed that when various species of plants were grown in either sand or water cultures containing selenate and sulfate in various ratios, sulfate reduced the toxicity to these plants and the accumulation of selenium. Her results suggested an antagonism between the two anions for uptake by the plant cells.

Postgate (1949) found competitive inhibitions of sulfate reduction by selenate in suspensions of Desulfovibrio desulfuricans. Further studies by Postgate (1952) indicated that sulfate and selenate were competitive antagonists for

growth and that sulfite and thiosulfate overcame selenate inhibition.

During investigation of the anti-metabolic action of selenate on sulfate reduction in Chlorella, which can use sulfate as the sole sulfur source, Shrift (1954) noted that sulfate and selenate fitted the criteria for antimetabolites. At any one ratio of sulfur to selenium, regardless of the absolute levels of each, the growth response was the same, indicating that the two analogues acted in a competitive manner. Analyses revealed that sulfate prevented the "absorption" of selenate.

Weissman and Trelease (1955) showed that sulfate and selenate were competitive antagonists for the growth of Aspergillus niger, as well as for the accumulation of selenium by the mold. They found that all sulfur containing amino acids tested lessened the selenate toxicity.

In studies with E. coli, Cowie and Cohen (1957) found that selenium partially replaced sulfur. Hydrolysates of the residual protein fraction incorporated radio-selenium, and a selenite-containing compound was synthesized by the organism. The compound had chromatographic properties similar to those of cysteine.

2. Effect of phosphate on selenite uptake and reduction

Leifson (1936) had already observed that phosphate lessened the toxicity of selenite to bacteria; however, while studying the effects of selenium and arsenic salts on the respiration of Saccharomyces cerevisiae, Pengra and Berry (1953) first reported an antagonism between phosphate and selenite. This antagonism was studied by Bonhorst (1955), who noted that inhibition of S. cerevisiae respiration by selenite was lessened in the presence of arsenite, arsenate or phosphate. Selenate inhibition was not affected.

Mahl and Whitehead (1961) demonstrated that with respiring cells of S. cerevisiae, phosphate suppressed radioactive selenite uptake, and selenite suppressed the uptake of radio-phosphate.

A selenite-phosphate antagonism in Candida albicans was observed by Falcone and Nickerson (1960, 1963). In the first study, these workers employed growing cells and they found that phosphate uptake was suppressed by selenite in a manner similar to that exerted by 2,4 dinitrophenol on phosphate assimilation by yeast cells. At the same time, phosphate inhibited the reduction of selenite. This antagonism indicated that selenite exerted its toxic action,

at least in part, by competing with phosphate at the sites of its absorption. In the second study, non-proliferating cells were used. The results parallelled those obtained with growing cells in that selenite uptake and reduction were markedly inhibited by phosphate. Inhibition was maximal at a phosphate concentration equimolar with that of selenite (0.01M).

Osborne and Stokes (1955) observed that the phosphate content in selenite containing enrichment media was of considerable importance. The inhibitory compound increased the phosphate requirement for the organism.

3. The role of amino acids in lessening the toxicity of selenite and selenate

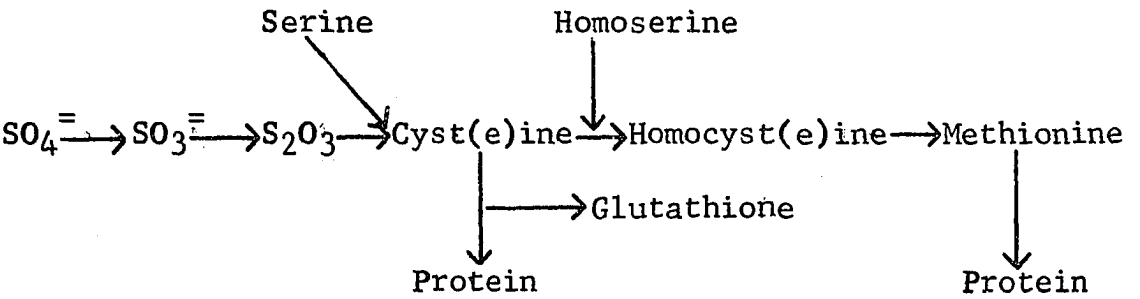
Fels and Cheldelin (1948) reported that selenate toxicity in yeasts could be partially reversed by l-methionine. For E. coli, however, cysteine and, to some extent, glutathione but not methionine was utilized in nullifying the inhibitory effect of selenate (Fels and Cheldelin, 1949a).

The incorporation of cystine into the selenite F enrichment medium as a means of overcoming deficiencies in some peptones was suggested by North and Bartram (1953). It was

recognized that even in a satisfactory medium the addition of cystine was beneficial since it decreased the toxicity of selenite.

Opienska-Blauth and Iwanowski (1952) observed that the growth of E. coli in Koser's citrate medium was not inhibited by selenite at concentrations up to 8 parts per million of selenium. Above this concentration, total growth was decreased, and metallic selenium was deposited after several hours of incubation. Pre-incubation with cysteine and histidine was found to reverse the growth inhibition of 8 parts per million of selenium (as 10^{-4}M selenite). No reversal with sulfate was observed.

Methionine was shown by Scala and Williams (1962) to enhance selenite toxicity in E. coli. On the basis of their findings they suggested that media for the isolation of bacteria from natural products would be more efficient in preventing growth of E. coli, if they contained l-methionine in addition to selenite. These investigators explained this phenomenon of increased selenite toxicity by considering the sulfur pathway in E. coli as postulated by Roberts et al. (1955).



According to Roberts et al. (1955), in a medium containing exogenous methionine, E. coli cells incorporated this amino acid directly into protein and biosynthesis of methionine ceased. Scala and Williams (1962) concluded that with selenite in the medium, the presence of methionine must have suppressed also the biosynthesis of seleno-methionine; this would have made $SeO_3^=$ available to oxidize certain functional groups of protein. The oxidation of sulfhydryl groups of enzymes may have rendered certain proteins biologically inactive, resulting in less total growth and in a reduced growth rate. It was also considered possible that seleno-cysteine or seleno-cystine made the cell protein partially or completely inactive.

4. The effect of selenium compounds on enzymes

Sodium selenite has been used for many years as an inhibitor of certain enzyme systems, particularly of those now known to be dependent on sulfhydryl groups for their activity. Woodruff and Gies (1902) observed inhibition of

alcoholic fermentation of yeast by selenite. Potter and Elvehjem (1936) noted a decrease in the oxygen uptake by yeast during the fermentation of sugars, but very little effect on respiration when lactate and pyruvate were used as substrates in the presence of selenite. Urease, but not arginase, catalase or acetyl choline esterase was inhibited by selenite (Wright, 1940). L-proline oxidase activity was found to be depressed by selenite (Bernheim and Klein, 1941), as was that of alkaline phosphatase (Hoitink, 1942), triose phosphate dehydrogenase (Bergstermann and Mangler, 1948), and succinic dehydrogenase (Stotz and Hastings, 1937).

5. The metabolism of selenium by microorganisms

Many studies have been undertaken to determine the fate of selenium in higher plants and animals. Bacterial reduction of selenium salts, particularly selenite, has been recognized for many years. Bacteria have been known to deposit elemental selenium when they are incubated under various conditions with inorganic selenium salts, but there are relatively few reports concerning the metabolism of selenium in microorganisms.

The effect of selenium salts on the yeast fermentation of glucose was studied by Moxon and Franke (1935). They

found that yeast while fermenting glucose could reduce selenite, but apparently not selenate, to intracellular metallic selenium, and that selenate was less toxic to fermentation than selenite. They were unable to obtain a reversal of selenite toxicity by additions of sulfate, sulfite, or thiosulfate.

Fels and Cheldelin (1949b) reported that the growth inhibition of the yeast, S. cerevisiae, by selenate was dependent on the sulfate concentration, and that one-half maximal growth was obtained with a sulfur to selenium ratio of one, in accordance with the postulate that sulfur and selenium are competitive antagonists. It was also found that l-methionine reversed the selenate toxicity, but the reversal was incomplete. That the reversal was not due to a chemical reaction between selenate and methionine, but to an enzymatic process, was believed to be evident from the fact that d-methionine was completely ineffective as a reversing agent (Fels and Cheldelin, 1948). It was further observed that thiamine and cysteine enhanced the reversing action of methionine, and that no dimethyl selenide was formed (Fels and Cheldelin, 1950). They postulated that selenate toxicity in yeast was partly due to a blocking of methionine synthesis,

and that the reversing action of methionine was not due to its conversion to sulfate nor to the methylation of selenium. It was concluded that the toxicity of selenate was a multiple effect.

A few studies have been conducted on the metabolism of selenite by molds. Zalokar (1953) showed that when selenite was added to a culture of Neurospora, metallic selenium was deposited inside the mycelia. The reduction of selenite was inhibited by cyanide, iodoacetate, carbon monoxide, and copper sulfate, and required sugar as an energy sourse. Challenger et al. (1953) found that the molds Aspergillus niger and Scopulariopsis brevicaulis could methylate selenite to dimethyl selenide.

Weissman and Trelease (1955) showed that all sulfur-containing amino acids tested were able to counteract partially or wholly the selenate inhibition of the growth of Aspergillus niger.

It was shown by Cowie and Cohen (1957) that seleno-methionine could effectively substitute for methionine. Exponential growth was obtained indicating that the cellular protein was fully functional.

A complete review of the different phases of the metabolism of selenium was given by Tuve (1958). In addition

to the review, the investigator presented findings of his own and concluded the following:

(1) For the E. coli strain used, the substitution of sulfur by selenium was not a quantitatively important process at subtoxic levels.

(2) There was compelling evidence that at least two different mechanisms for selenite toxicity (or metabolism) existed: (a) the reaction of selenite with sulfhydryl groups, and (b) the incorporation of selenium into analogues of sulfur compounds.

(3) At sub-toxic selenite levels, incorporation of selenium by E. coli grown in a sulfur-deficient glucose-salt medium containing radio-selenite was proportional to the increase of bacterial dry weight before, as well as after, depletion of sulfur in the medium. The rate of uptake of radioselenium increased considerably after sulfur depletion.

(4) Selenium was competitive with sulfur for the synthesis of cysteine, cystine, methionine, and their seleno-analogues. These analogues were incorporated into protein, and the observed incorporation of selenium was not thought to be due to either adaptation, adsorption, or the deposition of elemental selenium.

(5) At least two amphoteric compounds containing

selenium could be isolated from protein hydrolysates of E. coli cells. Seleno-methionine was identified, and the presence of selenocystine or of an analogue was postulated.

An extensive study of the effect of selenite on yeast was conducted by Falcone and Nickerson (1960, 1963) and Nickerson and Falcone (1963). The test organism was C. albicans, and in a series of experiments with growing and non-multiplying cells, these workers obtained, in addition to the above-mentioned selenite-phosphate antagonism, the following results:

(1) Selenite reduction was inhibited by methionine, formate, fluoride and dinitrophenol (DNP) as well as by certain sulfhydryl poisons. The inhibitory action of DNP could be counteracted with riboflavin 5'-phosphate (FMN). Reduction of selenite was markedly influenced by environmental conditions such as selenite concentration, pH, temperature, and time of incubation. The existence of an optimal pH for reduction of selenite by intact cells was thought to reflect the effect of pH on selenite permeation into the cells. A maximum concentration of the biselenite ion ($HSeO_3^-$) was attained at pH 4.9, since the dissociation constant for the first H of selenious acid is 3×10^{-3}

(pK_1=2.52), and for the second H, the dissociation constant is 5 x 10^{-8} (pK_2=7.30). Therefore, the biselenite ion was assumed to be the species of selenite permeating intact cells.

(2) Cell-free preparations capable of reducing selenite proved the enzymatic nature of selenite reduction. The activity was found to reside in a soluble, non-particulate fraction and to be complex, in that activity was lost upon dialysis but was restored by the addition of heat-stable, dialyzable components.

Fluharty and Sanadi (1960) showed that selenite appeared to be bound to protein through vicinal-thiol groups to be released from them as metallic selenium after accepting four electrons.

Recently, Scala and Williams (1963) compared selenite and tellurite toxicity in E. coli. They found that l-methionine also enhanced the toxicity of $TeO_3^=$ towards E. coli, suggesting that $TeO_3^=$ was reduced in the sulfur reduction pathway in a manner similar to that of $SeO_3^=$. Enhancement of tellurite toxicity could, however, be produced by a wide variety of sulfur compounds. Such compounds included those which served as complete or partial sources of sulfur for E. coli in addition to those not metabolized by

the organism. This difference between selenite and tellurite toxicity demonstrated that a sulfur-tellurite interaction took place, which had an effect on E. coli that was not comparable to any sulfur-selenite interaction.

III. EXPERIMENTAL

A. Materials and Methods

1. Growing cell experiments

a. Organisms used Laboratory strains of E. coli (ISU-41), P. vulgaris (ISU-37c), and S. thompson (ISU-86-2) were maintained as stock cultures on nutrient agar slants.

b. Medium employed and determination of growth The basic culture medium for determining selenite resistance of the organisms consisted of a tryptone-phosphate broth containing tryptone* (5.0 g/l), dipotassium phosphate (7.5 g/l), and monopotassium phosphate (2.5 g/l). The pH was 7.0 ± 0.1. Twenty-ml samples in 15 x 150 mm screw cap Pyrex test tubes were used routinely, except where noted. Each tube received 5 ml of triple strength broth. Selenite solutions of required concentrations were prepared as 15-ml samples from a 5% stock solution. The broth and selenite solution were sterilized separately and mixed aseptically after cooling. The tubes were inoculated with 0.1 ml of actively growing broth cultures of the three organisms and incubated at 37°C.

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*Bacto-Tryptone, Difco Laboratories, Detroit, Mich.

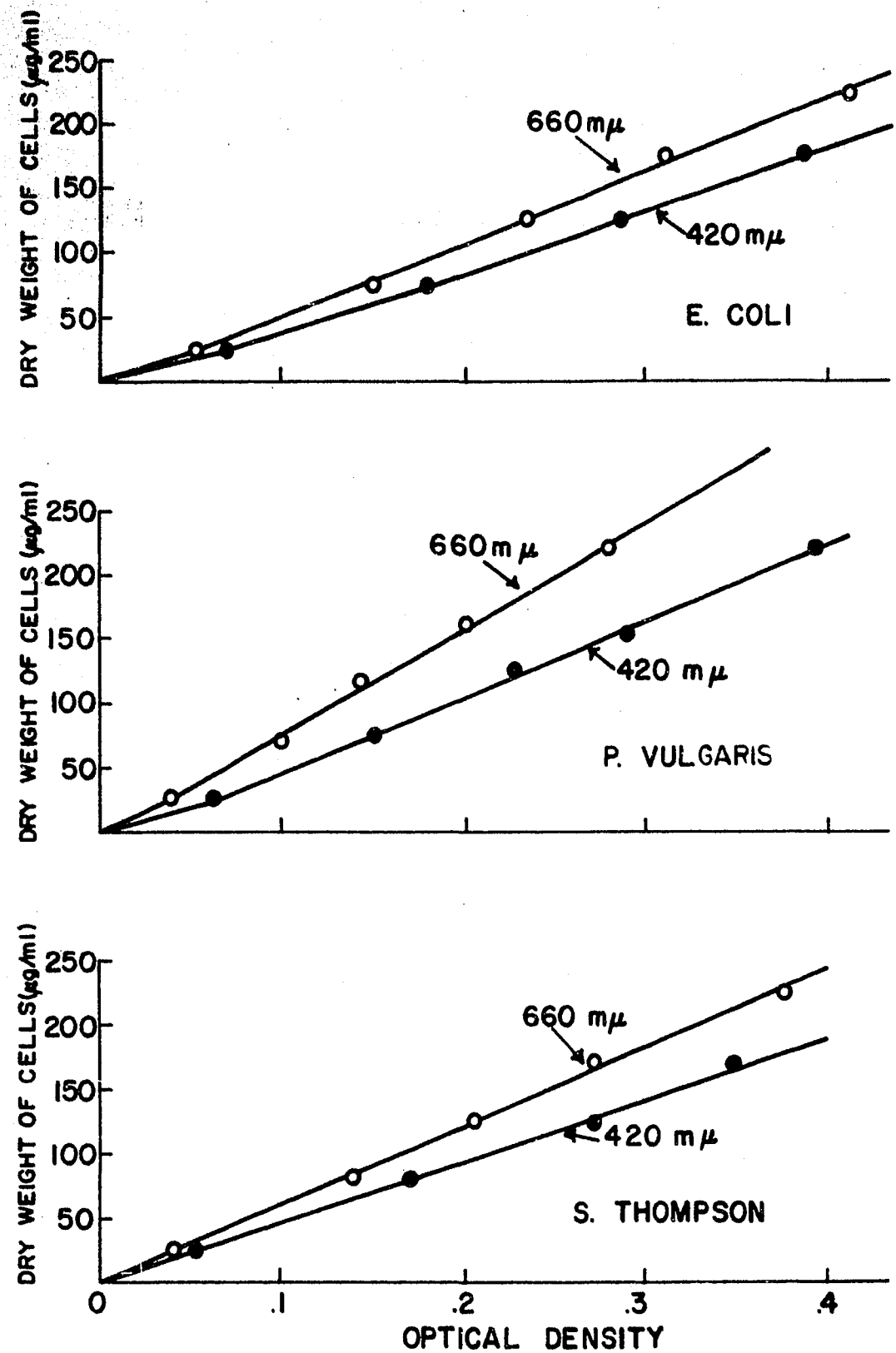
Plate counts were made at 0, 12, 24 and 48 hours; nutrient agar was the plating medium.

c. Addition of inorganic ions to the selenite broth To study the effect of selected inorganic ions on the toxicity of selenite to the organisms, phosphate, sulfate and sulfite were added to the culture medium containing selenite to give final salt concentrations of 10, 5 and 0.15%, respectively. The phosphate and sulfate were added to the broth prior to autoclaving; the sulfite, however, was sterilized separately to avoid oxidation.

d. Addition of amino acids Cystine, methionine, and histidine were incorporated into selenite broth to test for possible reversal of selenite toxicity. Sterile solutions of the amino acids were added to the medium to give a final concentration of 100 μg/ml.

e. Estimation of selenite reduction The reduction of selenite to red, metallic selenium was estimated by the method used by Falcone and Nickerson (1960) except that a Bausch and Lomb spectrophotometer instead of a Klett-Summerson colorimeter was used. Standard graphs were plotted (Figure 1) from optical density values obtained at 420 m μ and 660 m μ for various dilutions of cell suspensions of known dry weights of the three organisms grown in the absence

Figure 1. Optical densities of cell suspensions of *E. coli*, *P. vulgaris*, and *S. thompson*


DRY WEIGHT OF CELLS (µg/ml)
250
200
150
100
50
660mµ
420mµ
E. COLI
DRY WEIGHT OF CELLS (µg/ml)
250
200
150
100
50
660mµ
420 mµ
P. VULGARIS
DRY WEIGHT OF CELLS(µg/ml)
250
200
150
100
50
660 mµ
420 mµ
S. THOMPSON
0
.1
.2
.3
.4
OPTICAL DENSITY

of selenite.

The cell mass (dry weight) was determined gravimetrically for each of the three species. The percent dry weights were found to be as follows: for E. coli, 23.9%, for P. vulgaris, 22.9%; for S. thompson, 23.2%. Cell suspensions containing known wet weights/ml were related to optical densities and from the determined percent dry weight, the μg dry weight/ml was calculated.

From the observed absorbancy values of the test samples at 660 m μ, (due almost entirely to turbidity arising from culture growth), the corresponding values at 420 m μ were located on the standard curves. Any additional absorbancy at 420 m μ observed in the cultures grown in the presence of selenite was taken as a measure of selenite reduction. The corrected optical density values obtained at 420 m μ were compared with a standard reduction curve to obtain a quantitative estimate of reduction. The standard reduction curve (Figure 2) was prepared from a series of standard solutions containing from 0 to 90 μg sodium selenite/ml. The selenite was reduced to red, metallic selenium with 5% ascorbic acid, and sterile tryptone phosphate broth was added to each standard to compensate for the natural coloring of the culture medium.

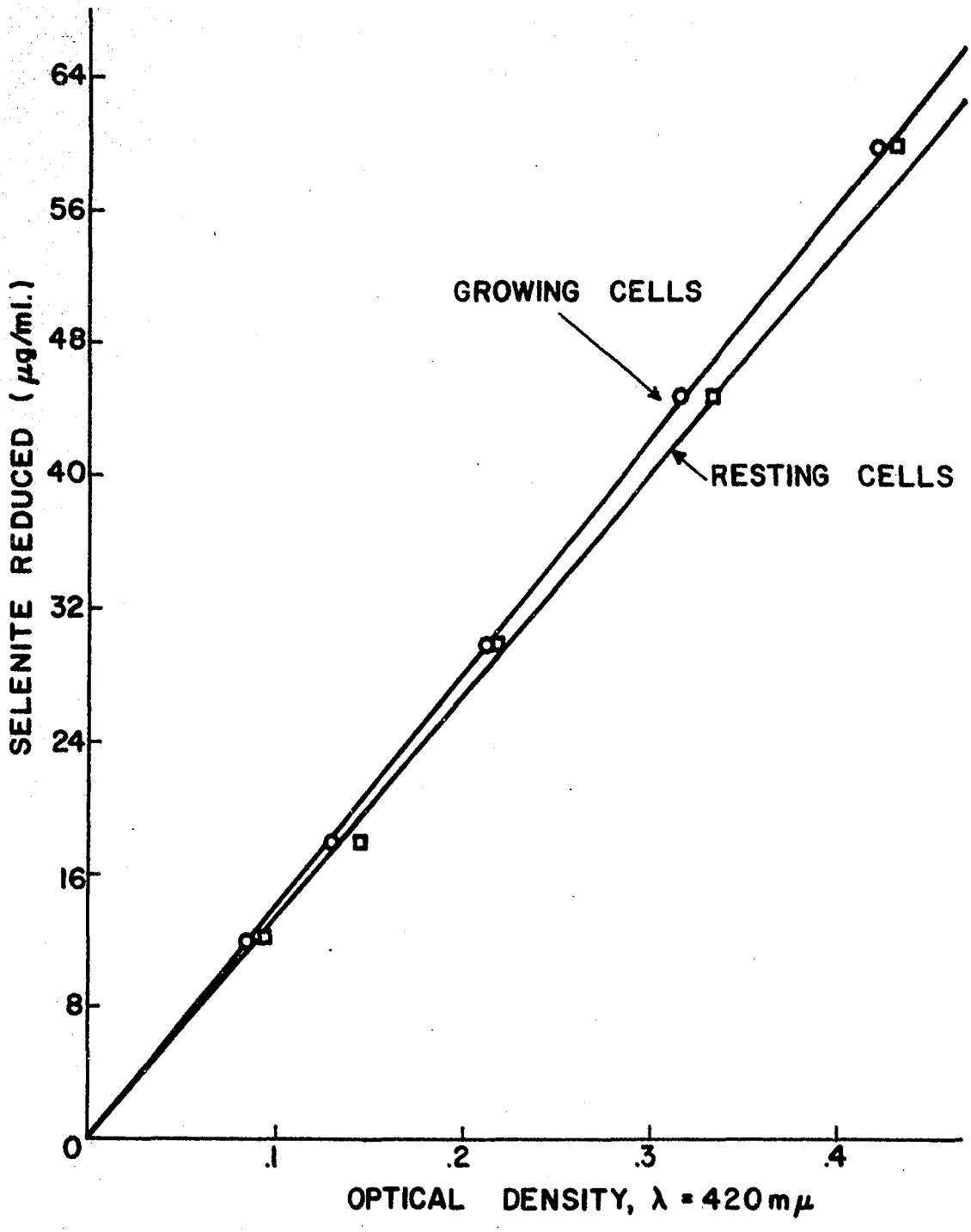


Figure 2. Calibration curves for estimation of selenite reduction

f. Determination of selenite uptake in relation to cell mass Total selenite uptake by growing cells was determined in the following manner: 5-ml aliquots of the cultures containing radioactive selenite were withdrawn at appropriate intervals during growth and filtered through Millipore (porosity 0.22 μ) filters to collect the organisms. The collected cells were washed with four 10 ml volumes of distilled water to remove soluble radioactive material adhering to the cells and to wash out the unbound radioactive compounds. The filters were dried, placed on aluminum planchets and the radioactivity was counted with a Picker Proportional Flow Counter equipped with an ultra thin window (less than 0.15 mg/cm^2).

2. Resting cell experiments

a. Microbiological procedures For mass growth, each of the three organisms was grown in brain heart infusion broth at 37°C for 48 hours on a rotary shaker, harvested by centrifugation and washed six times with physiological saline. The cell density used was 10 mg/ml (dry weight); stock suspensions were prepared by suspending cells to give the equivalent of 1 g dry weight in water to make 40 ml.

b. Estimation of reduction and uptake of selenite Test tubes containing 10 mg cells/ml (dry weight) in a total of 5 ml of the appropriate radioactive reaction mixtures

containing any ingredients to be tested, were incubated at 37°C for 180 minutes. The tubes were centrifuged for 5 minutes at 3400 x g and 1/2 ml of the supernatant was placed on a planchet, dried and the radioactivity was counted.

The extent of reduction was determined in the following manner: 1/2 ml of the suspension was diluted to 5 ml and its optical density read at 420 m μ. The absorbancy at this wave length was due chiefly to the formation of red selenium; however, in order to correct for non-specific changes in turbidity during incubation, the optical density was also determined at 660 m μ, (Falcone and Nickerson, 1960). An incubated selenite-free cell suspension served as a blank. Corrected optical density readings were compared to μg of selenium by reference to a calibration curve constructed in the same manner as for growing cells (Figure 2). Standards were prepared from cell suspensions having densities of 1 mg/ml (dry weight), by adding known amounts of selenite and reducing with 5% ascorbic acid.

3. Incorporation of selenium into cell fractions

a. Growth of organisms The same organisms and the same basal medium as those described previously were used. The selenite ($NaHSeO_3$) concentration was 0.1%, containing

Se^{75} to give a specific activity of 10^4 c/m/ml in a total volume of 1,000 ml.

The inoculated broth was incubated at 37°C for 48 hours on a rotary shaker. The cells were then centrifuged and washed six times with physiological saline so that the supernatant was free from radioactivity.

b. Fractionation The procedures suggested by Roberts et al. (1955) were generally adhered to for routine fractionation of cells. (1) The washed cells from 1,000 ml medium, and weighing approximately 100 mg, were suspended in 10 ml of 5% trichloroacetic acid in a 50 ml plastic centrifuge tube and held for 30 minutes at 5°C. The suspension was then centrifuged yielding the cold-TCA soluble fraction. (2) The precipitate was suspended in 10 ml 75% ethanol. After 30 minutes at 37°C the suspension was centrifuged. The supernatant contained the alcohol-soluble proteins and lipids. (3) The precipitate was suspended in 10 ml of an ethanol-ether (50-50) mixture. The suspension was maintained at 37°C for 30 minutes and then centrifuged to separate the remaining lipids of the cells. (4) The precipitate was suspended in 10 ml of 5% TCA and kept in boiling water for 30 minutes. The suspension was centrifuged yielding the hot-TCA soluble material (nucleic acids and their derivatives). (5) The

remaining precipitate was washed free of residual TCA by suspending in acidified alcohol and centrifuging followed by washing in ether and centrifuging. The washings were discarded. The precipitate was the principal cell protein.

c. Hydrolysis Enzymatic hydrolysis only was employed because of the labile nature of organoselenium compounds. The hydrolytic procedure utilized three different enzymes (Tuve, 1958) and was as follows: Two 20 mg samples of the residual protein were incubated for 30 hours at 37°C with 2 mg of pepsin in 5 ml of 0.06 N hydrochloric acid. The solutions were then neutralized to approximately pH 7.5 with dilute sodium hydroxide and again incubated for 30 hours at 37°C after the addition of 15 mg pancreatin to each sample. Four mg erepsin were then added and incubation was continued for an additional 48 hours at 37°C. The hydrolysates were centrifuged to precipitate the insoluble selenium, and the clear supernatants were combined and transferred to an ion exchange resin.

d. Ion exchange columns The ion exchange resin used was Dowex 50, a cation exchanger in the hydrogen form. The resin was prepared by treating commercial Dowex-50, 200-400 mesh, 8% cross-linked, with 6N hydrochloric acid until the supernatant was colorless. The resin was then washed to

approximately pH 6 with distilled water and poured as a slurry into 10 ml burettes.

e. Chromatographic procedures

1) Column chromatography 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 10 ml portions of 1.5N and 6N hydrochloric acid. The fractions containing the highest radioactivity were dried at 50°C. The residue was resuspended in water and redried 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 an aliquot was withdrawn for investigation by paper chromatography and radioautography.

2) Paper chromatography Descending, one-dimensional chromatography was employed throughout, using Whatman No. 1 paper (10 x 45 cm). The points of origin were located and the material to be chromatographed was spotted. The spots were allowed to dry, the chromatograms placed in chromatographic jars and irrigated with solvent (isopropyl alcohol, formic acid, water; 70:10:20; Roberts et al., 1955) at room temperature. 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 minutes 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. The irrigated and dried chromatograms containing the known amino acids were developed in a 0.25% $^{w}/_{v}$ solution of ninhydrin in acetone containing 7% $^{v}/_{v}$ glacial acetic acid and finally heated for 15 minutes at 45°C (Mizell and Simpson, 1961). The presence of selenite was determined by dipping the chromatogram in acetone containing 10% $^{v}/_{v}$ of an aqueous 10% solution of ascorbic acid.

Ninhydrin was also used for positive identification of seleno-amino acids by the "fingerprint" method (Tuve and Williams, 1957). The spot on the chromatogram containing the radioactive compound to be identified was cut out and eluted with water into a test tube. The apparatus used was similar to that described by Aronoff (1956). The eluted material was mixed with a quantity of known carrier (non-radioactive)

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*The author wishes to thank Dr. Klaus Schwarz of the National Institutes of Health for samples of the three synthetic seleno-amino acids.

compound suspected to be identical with the unknown, and then chromatographed. The dried chromatogram was radioautographed, as described in the following paragraph, and then developed in either the ninhydrin or the ascorbic acid solution to detect the known carrier material. If the spot on the radioautograph produced by the radioactivity of the unknown material, and the colored spot produced by the chemical test superimposed exactly, the two compounds were identical. If the two compounds were not identical, the two spots did not superimpose and appeared "pushed aside" by each other. The radioactive material must not have contributed to the chemical test employed for the detection of the carrier material.

f. Radioautography 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 were exposed to the radioactive material on the chromatograms. The corners of the chromatograms were labelled with radioactive ink; the radioactive inkspots served to indicate positions of the radioautographs.

After exposure, the films were developed in Kodak D-19 and treated in acid fixer (hypo) until clear. The developed films were then washed in running water and dried.

B. Results and Discussion

1. Growing cells

a. Comparative tolerances to selenite and effect of additives on growth The different degrees of resistance to increasing concentrations of sodium selenite exhibited by the three bacterial species were clearly demonstrated in Figure 3.

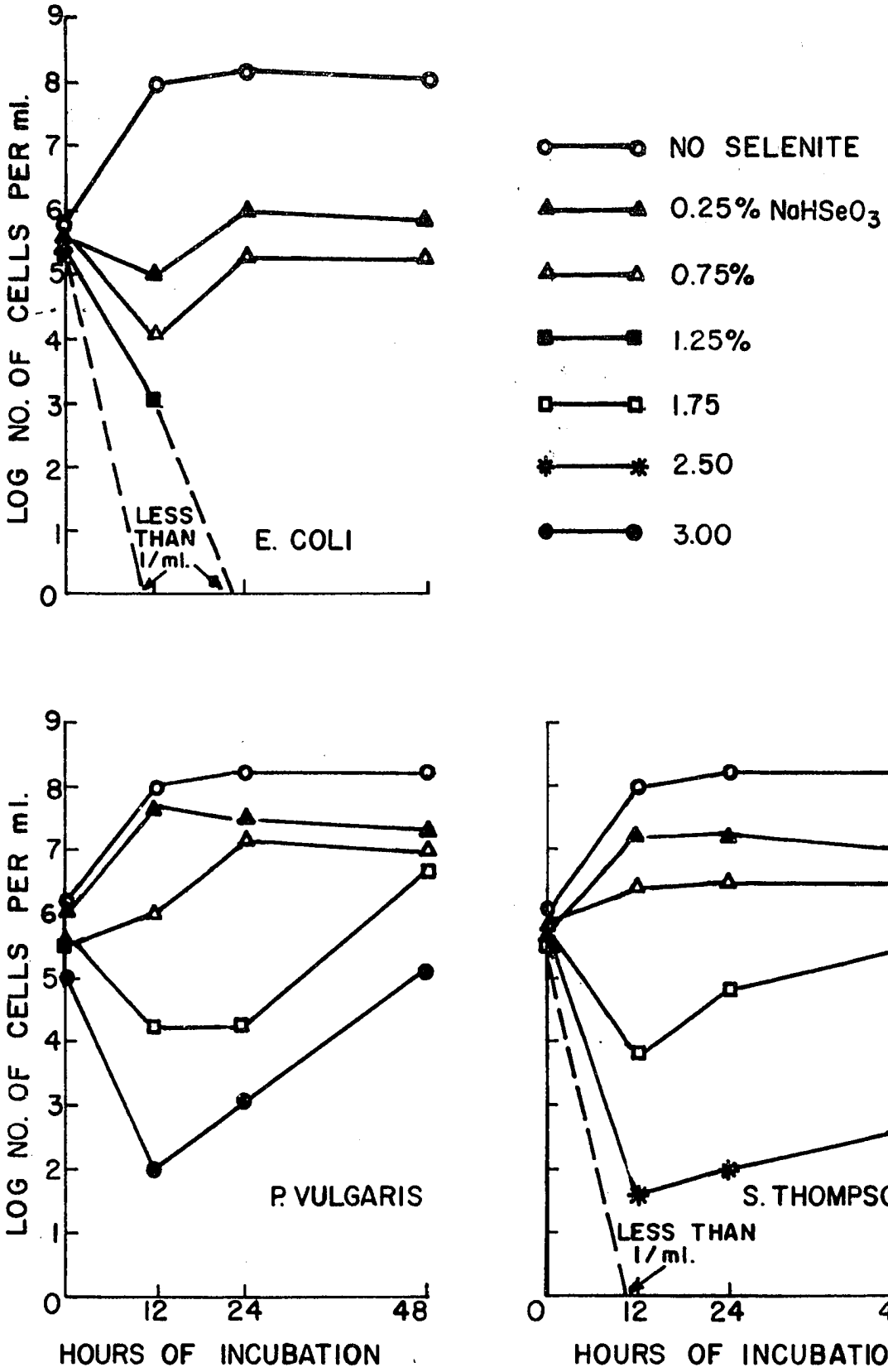
A concentration of 1.25% $NaHSeO_3$ resulted in complete inhibition of E. coli, and at a concentration as low as 0.25% sodium selenite there was a pronounced lag; the number of viable cells at the end of 48 hours was only about as great as the inoculum.

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

These growth curves served to illustrate the effect of

Figure 3. Effect of selenite concentrations on growth of *E. coli*, *P. vulgaris*, and *S. thompson*


LOG NO. OF CELLS PER ml.
0
1
2
3
4
5
6
7
8
9
LESS THAN 1/ml.
E. COLI
NO SELENITE
0.25% NaHSeO3
0.75%
1.25%
1.75
2.50
3.00
P. VULGARIS
S. THOMPSON
12
24
48
HOURS OF INCUBATION

sodium selenite on the three organisms in a liquid enrichment medium. The pronounced lag (Phoenix phenomenon) in the early 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.

S. thompson and P. vulgaris showed similar growth patterns in the tryptone-phosphate broth with concentrations of $NaHSeO_3$ as great as 2.5%. Nevertheless, there may be differences in the mechanism of the action of selenite. Also, the addition of selected inorganic ions or amino acids to the selenite medium may have different or differentiating effects on the two organisms. It was thought that the effects of the "additives" would give some indication of possible differences in the action of selenite on resistant and susceptible microorganisms.

Figures 4 to 6 show the effect of three inorganic ions on the toxicity of selenite towards the organisms used. The sulfur salts were chosen since they could be considered possible metabolic competitors; phosphate was included in the study because various workers have shown a relationship

Figure 4. Effect of 10% phosphate on growth of *E. coli*, *P. vulgaris* and *S. thompson* in a selenite containing medium

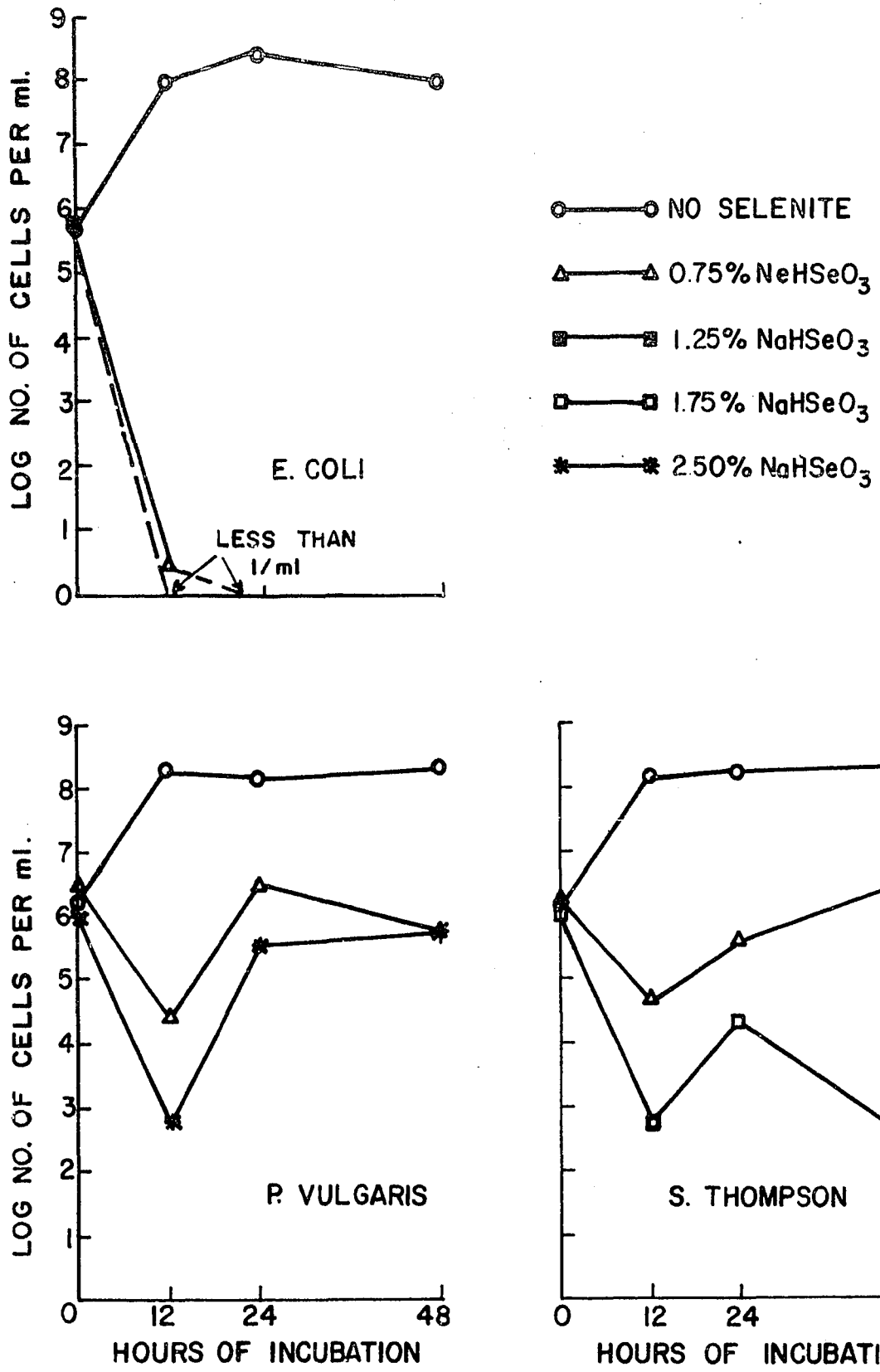

LOG NO. OF CELLS PER ml.
9
8
7
6
5
4
3
2
1
0
E. COLI
LESS THAN
1/ml
NO SELENITE
0.75% NeHSeO3
1.25% NaHSeO3
1.75% NaHSeO3
2.50% NaHSeO3
LOG NO. OF CELLS PER ml.
P. VULGARIS
S. THOMPSON
0
12
24
48
HOURS OF INCUBATION
HOURS OF INCUBATION

Figure 5. Effect of 5% sulfate on growth of *E. coli*, *P. vulgaris* and *S. thompson* in a selenite containing medium

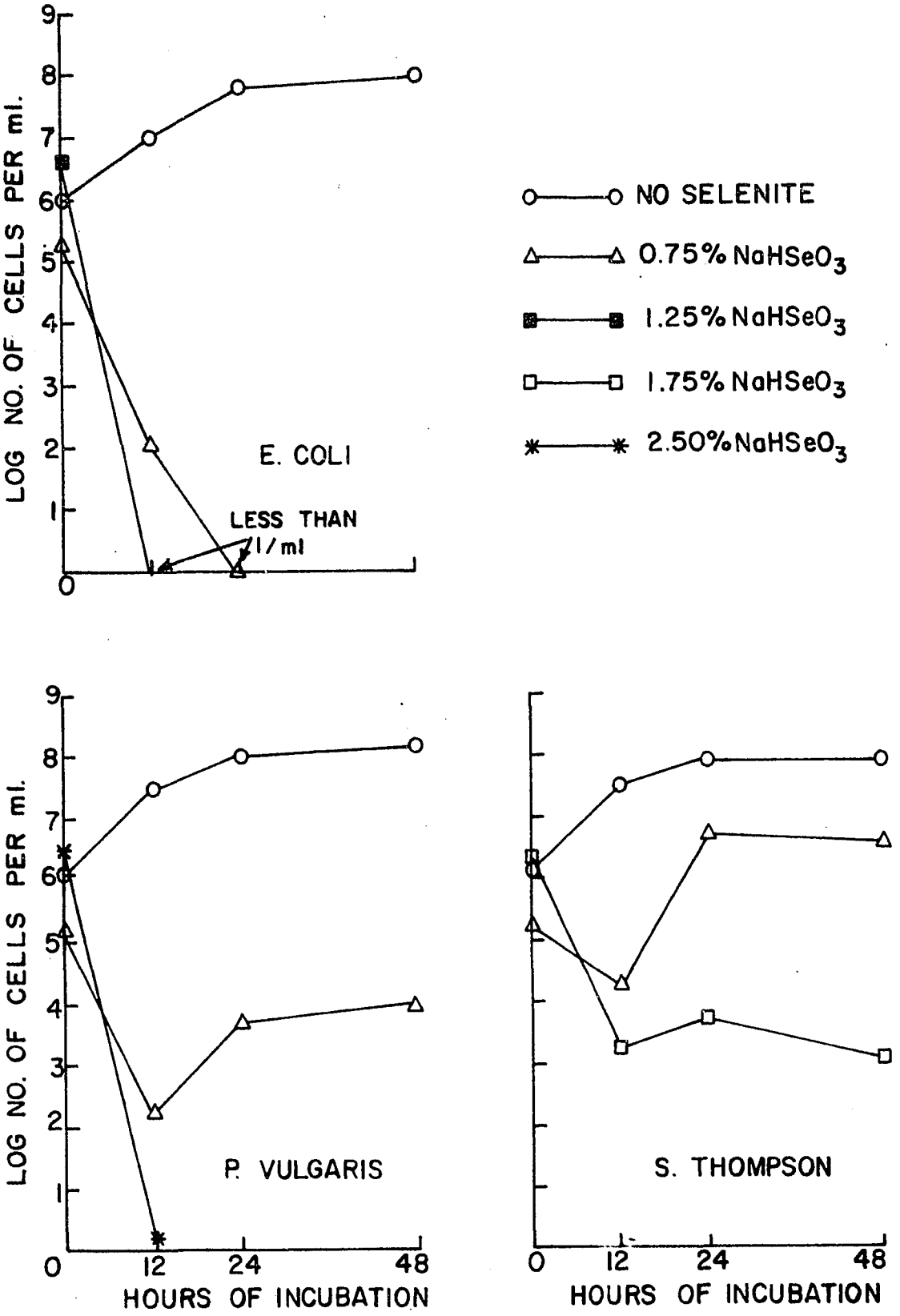
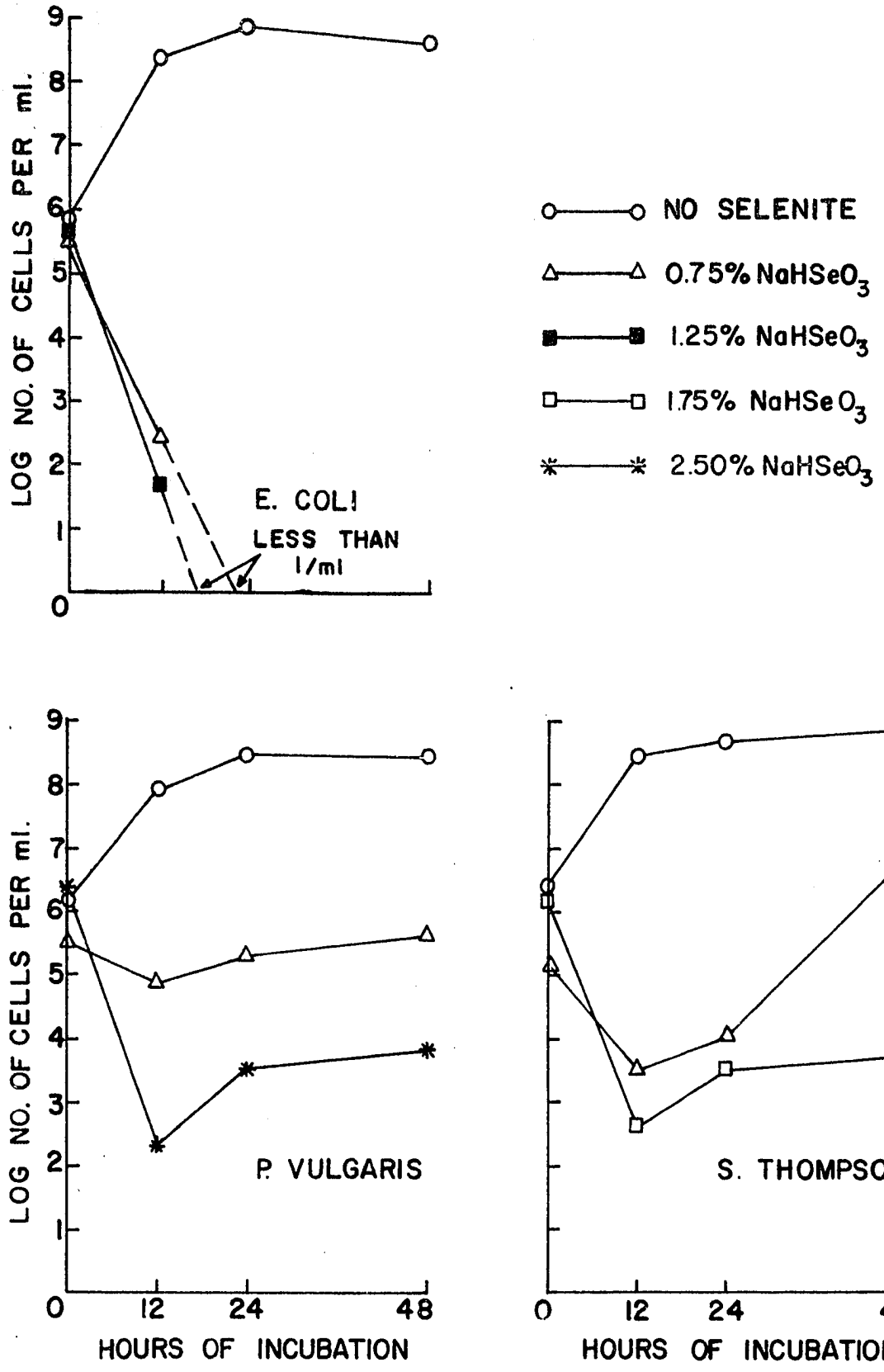

LOG NO. OF CELLS PER ml.
9
8
7
6
5
4
3
2
1
0
E. COLI
LESS THAN 1/ml
NO SELENITE
0.75% NaHSeO3
1.25% NaHSeO3
1.75% NaHSeO3
2.50% NaHSeO3
P. VULGARIS
S. THOMPSON
0
12
24
48
HOURS OF INCUBATION

Figure 6. Effect of 0.15% sulfite on growth of *E. coli*, *P. vulgaris* and *S. thompson* in a selenite containing medium


LOG NO. OF CELLS PER ml.
0
1
2
3
4
5
6
7
8
9
E. COLI
LESS THAN
1/ml
NO SELENITE
0.75% NaHSeO3
1.25% NaHSeO3
1.75% NaHSeO3
2.50% NaHSeO3
P. VULGARIS
S. THOMPSON
0
12
24
48
HOURS OF INCUBATION

between phosphate uptake and selenite toxicity in yeast (Bonhorst, 1955; Mahl and Whitehead, 1961). Selenite was shown to interfere with the uptake of phosphate from the culture medium but high concentrations of phosphate suppressed the amount of selenite taken up by the cells.

The effect of the added inorganic salts was investigated with two different levels of selenite. The low selenite concentration was 0.75% for all three organisms. This concentration did not totally inhibit any of the three species but caused variations in growth rates. The high concentration was either completely inhibitory or permitted only marginal growth. The concentrations of the three added ions chosen were the maximum amounts that would permit unrestricted growth of the three organisms in the absence of selenite.

The toxic effect of selenite was not lessened for any of the three organisms by the addition of 10% phosphate (Figure 4); in fact, the selenite toxicity was markedly increased at the low selenite level for E. coli, P. vulgaris and S. thompson. There was little change in the growth pattern at the high concentration of selenite as compared to the control without added phosphate.

The addition of 5% sulfate also generally enhanced the toxicity of selenite for the three bacterial species (Figure

5). P. vulgaris was most severely affected; at the high selenite concentration, the viable cell count was reduced to less than 10 organisms per milliliter at 12 hours and no recovery occurred.

Sulfite at a concentration of 0.15% markedly affected the growth of S. thompson at the low selenite level (Figure 6). Here, the Phoenix phenomenon was strongly exhibited, that is, after a substantial decrease in viable counts during the first 12 hours there was a sharp rise after 24 hours. The growth patterns for E. coli and P. vulgaris differed little from those with added sulfate.

The results obtained with the added phosphate do not agree with the findings of Bonhorst (1955) for yeast, and they do not support the view held by Leifson (1936) that phosphate generally lowers the selenite toxicity towards microorganisms. The high phosphate concentration probably lowered the uptake of selenite thereby making more of the selenium salts available for the oxidation of essential sulfhydryl groups.

The increase in toxicity with the added sulfate and sulfite is in agreement with Leifson's conclusions; however, on the basis of competitive inhibitions of sulfur utilization

by selenite, an increased sulfur content of the medium should reverse the selenite toxicity. It was stated by Tuve (1958) that sulfate depressed the toxicity of selenite when supplied in "low" concentrations when the dominant phenomenon was the incorporation of selenium into analogues of sulfur compounds. At high levels of selenite, however, when the reaction of selenite with essential sulfhydryl groups became the basis of toxicity, added sulfate did not lessen selenite toxicity.

Under certain conditions, it is well possible that the effect of high levels of sulfate on selenite toxicity is similar to that described for high phosphate concentrations.

In addition to the inorganic salts, the three amino acids, cystine, methionine, and histidine, were tested at selenite levels which strongly inhibited the three organisms in the basal medium. Cystine and methionine were used because they are sulfur-containing amino acids and can conceivably block the toxic action of selenite on the cell protein. The view has been expressed that one possible way by which selenite exerts its toxic effect is in the form of seleno-analogues of sulfur-containing amino acids (Tuve, 1958). Histidine was included because Opienska-Blauth and Iwanowski (1952) found that it had a definite sparing action on the growth of E. coli in Koser's citrate medium containing

selenite. The effect of histidine on selenite is believed to be similar to its influence on the cobalt blockage of imidazol groups of some enzymes. The action of histidine is such that there is a double coordination of cobalt with histidine-imino- and histidine-amino nitrogen (Burk et al., 1946). With most other amino acids only one proton is replaced by cobalt.

According to Cowie and Cohen (1957) the anti-inhibitory action of cystine is due to the fact that (in E. coli) seleno-cysteine and seleno-cystine are synthesized to some extent from selenite. These two amino acid analogues, however, are considered not adequate for the synthesis of active protein, because either no Se-S or Se-Se bridges are formed, or proteins containing Se-Se or Se-S linkages instead of S-S bridges are impaired in their normal catalytic activity. This may be due to possible changes in the spatial configuration of the α helix of the protein when a sulfur atom is replaced by selenium. Added cystine would conceivably suppress the biosynthesis of seleno-cysteine and seleno-cystine thereby lessening the toxic action of selenite. It is also possible, according to Cowie and Cohen (1957), that the responsible enzymes (in E. coli) are incapable of transforming seleno-cysteine and seleno-cystine into seleno-methionine

which, not only is considered non-toxic, but could serve as the sole source of "methionine" for the growth of a methionine-requiring mutant (Cowie and Cohen, 1957). The role of exogenous methionine may be two-fold: if methionine can be used as the sole source of sulfur as in yeast (Fels and Cheldelin, 1948), the addition of this amino acid to the growth medium should counteract the inhibitory effect of selenite as a competitor with sulfate. On the other hand, if methionine is synthesized from cysteine or cystine, added methionine may actually increase the toxicity of a given selenite concentration as was reported by Scala and Williams (1962). The presence of methionine in a selenite-containing medium must prevent the biosynthesis of the innocuous seleno-methionine and more labile Se-S or Se-Se linkages will be formed due to the accumulation of seleno-cysteine or seleno-cystine. Furthermore, there may be a greater concentration of $SeO_3^=$ available to oxidize certain functional groups of proteins (Tuve, 1958) resulting in the inactivation of some enzymes.

The counts shown in Table 1 indicate that with a high concentration of selenite (1.25%) none of the three amino acids reversed the selenite toxicity for E. coli. It must be assumed that at this selenite concentration, inhibition of

Table 1. Effect of amino acids on growth of test organisms in the presence of selenite

	Cells per ml x 10,000					
	E. coli		*P. vulgaris*		*S. thompson*	
Selenite concentration	1.25%		3.00%		2.50%	
Time (h)	0	48	0	48	0	48
	(x 10^0)					
Control	60	<1	10	17	30	0.5
Cystine	45	<1	20	420	35	4
Methionine	53	<1	18	200	40	0.1
Histidine	50	<1	10	95	38	15

growth was complete before any sparing action could take effect.

The growth of *P. vulgaris*, which was not totally inhibited by 3.00% sodium selenite, was enhanced by all three amino acids. Cystine caused a 25-fold increase in numbers indicating a competition between formed seleno-cystine and added cystine for protein synthesis. The fact that, with methionine added, the counts obtained were more than 10 times

as high as for the control suggests that the exogenous methionine requirement of this organism is relatively high; that is, breakdown of methionine (seleno-methionine) to cystine (seleno-cystine) predominates over the reverse (synthetic) pathway.

The results for S. thompson indicate a selenite pathway similar to that suggested for E. coli by Roberts et al. (1955). The addition of cystine yielded a count approximately seven times as high as that of the control. Methionine depressed growth considerably, whereas histidine increased the final count more than 20 times over the control.

b. Kinetic aspects of selenite incorporation

Studies of selenite utilization by the three different organisms were conducted with two widely different selenite concentrations to test if 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 non-toxic (50 μg/ml) as well as the selectively inhibitory (7500 μg/ml), the same general pattern of total utilization and net uptake of selenite was obtained (Figures 7 and 8). At the low selenite concentration, there was a massive uptake of selenium by E. coli within the first

Figure 7. Net uptake of selenite by growing cells of *E. coli*, *P. vulgaris* and *S. thompson* at a selenite concentration of 50 μg/ml in tryptone phosphate broth

The increase in dry weight was determined by relating optical density values to the standard curves at 660 m μ given in Figure 1

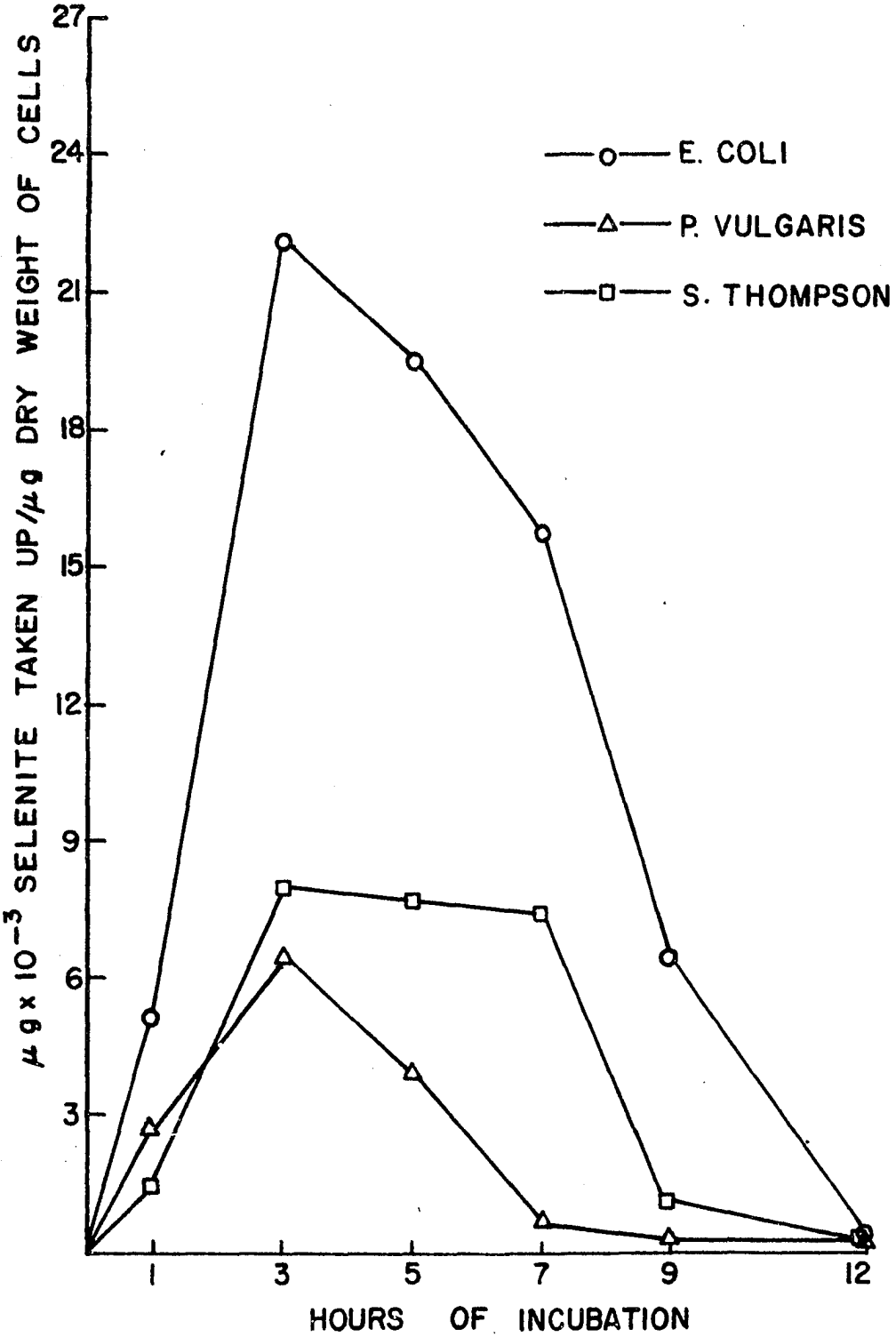
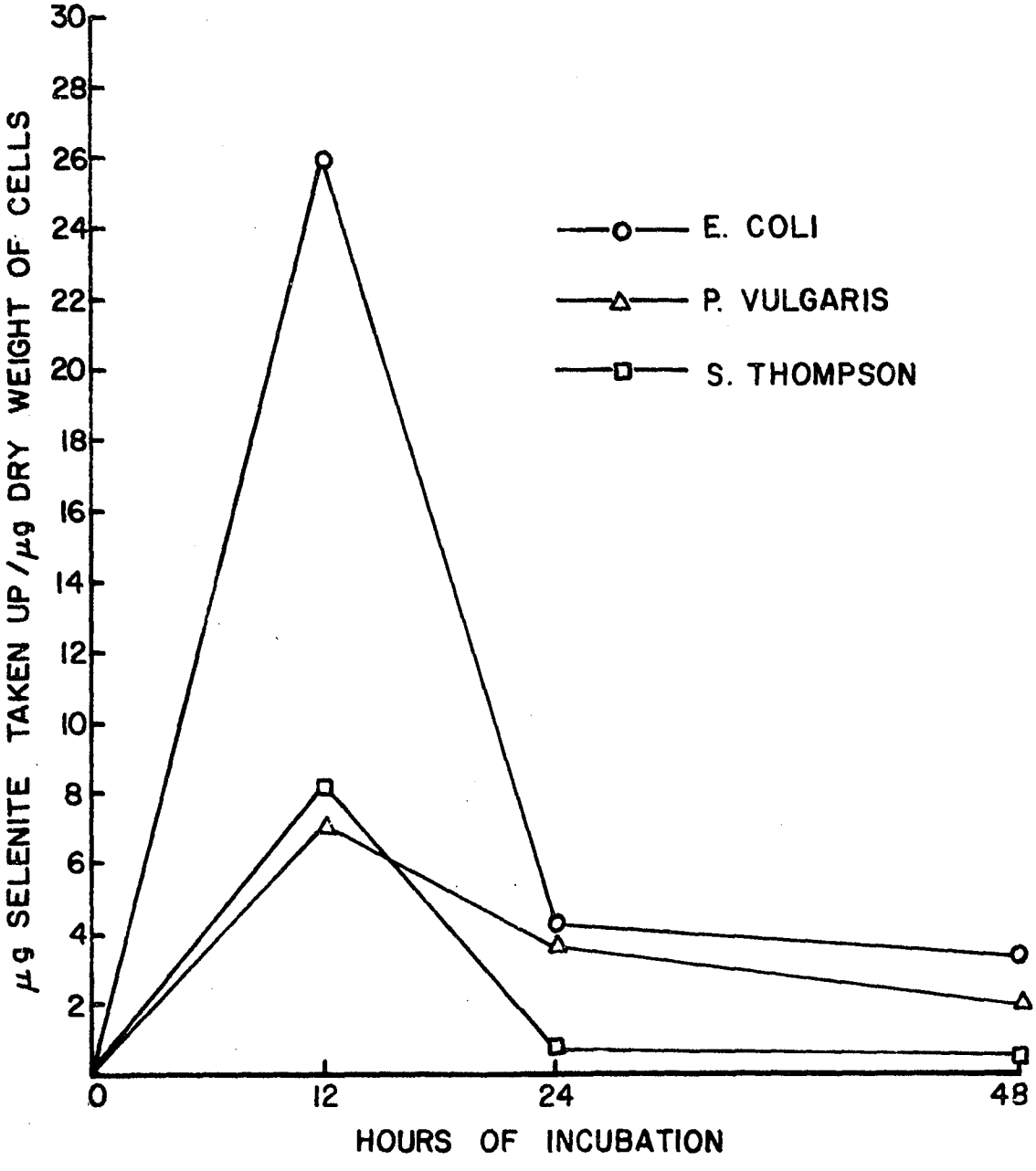

27
24
21
18
15
12
9
6
3
μg x 10⁻³ SELENITE TAKEN UP/μg DRY WEIGHT OF CELLS
E. COLI
P. VULGARIS
S. THOMPSON
1
3
5
7
9
12
HOURS OF INCUBATION

Figure 8. Net uptake of selenite by growing cells of *E. coli*, *P. vulgaris* and *S. thompson* at a selenite concentration of 7500 μg/ml in a tryptone phosphate broth

The increase in dry weight was determined by relating optical density values to the standard curves at 660 m μ given in Figure 1


30
28
26
24
22
20
18
16
14
12
10
8
6
4
2
0
12
24
48
μg SELENITE TAKEN UP / μg DRY WEIGHT OF CELLS
HOURS OF INCUBATION
E. COLI
P. VULGARIS
S. THOMPSON

three hours of incubation time (Figure 7). The uptake by the other two organisms during the same time was less than half of that taken up by E. coli. The same patterns were exhibited at the high selenite concentrations (Figure 8). The susceptible E. coli, which sustained a considerable lag at this concentration (see Figure 3) during the first 12 hours, took up 26 μg 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 selenium, respectively, per μg dry weight of cells.

The relationship between total utilization, reduction and net uptake of selenite by the three microorganisms was demonstrated more clearly in Figure 9. At the low selenite concentration utilization of selenite was linear with increasing dry weight of cells after one hour* of incubation time for S. thompson and P. vulgaris. With E. coli, after a rapid uptake of selenium between the first and third hour of incubation time, total selenite uptake also became linear. After seven hours, net uptake of selenite decreased sharply while the rate of reduction increased. This was true for all

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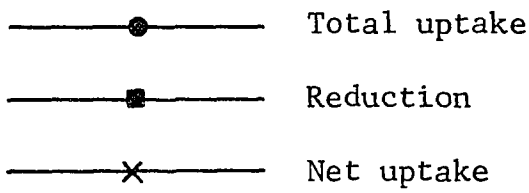
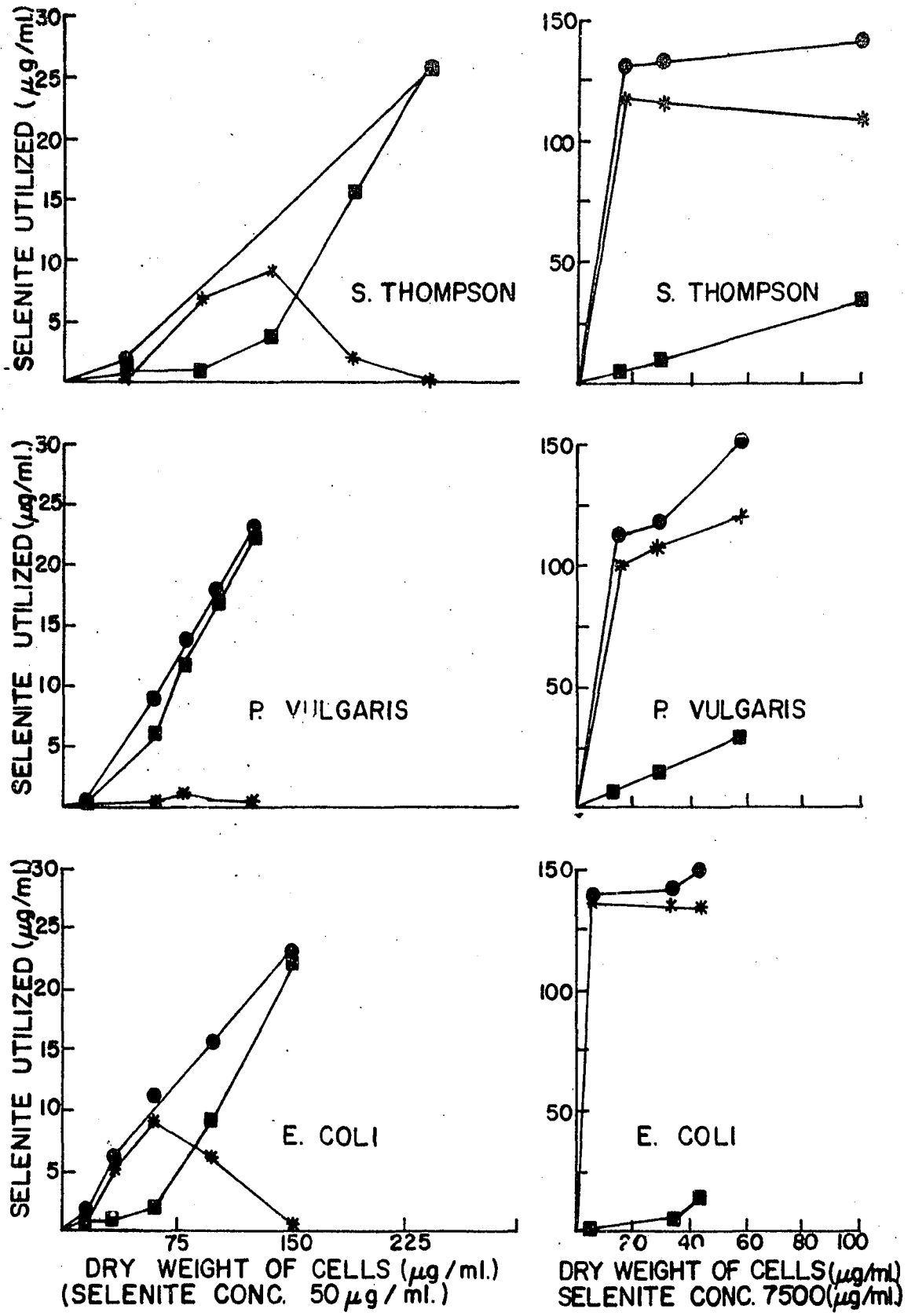
*Increase in dry weight during incubation time from Figure 1.

Figure 9. Selenite utilization by E. coli, P. vulgaris, and S. thompson

Measurements made for the low selenite concentrations at 1, 3, 5, 7, 9, 12 hours of incubation and at 12, 24, and 48 hours of incubation for the high selenite concentration




SELENITE UTILIZED (µg/ml)
S. THOMPSON
S. THOMPSON
SELENITE UTILIZED (µg/ml.)
P. VULGARIS
P. VULGARIS
SELENITE UTILIZED (µg/ml.)
E. COLI
E. COLI
DRY WEIGHT OF CELLS (µg/ml.)
(SELENITE CONC. 50 µg/ml.)
DRY WEIGHT OF CELLS (µg/ml)
SELENITE CONC. 7500 (µg/ml.)

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 approximated each other, especially for E. coli, which showed very little reduction for the first 24 hours. 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 hours. 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.

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 actively metabolized in the synthesis of seleno-analogues of sulfur-amino acids. Tuve and Williams (1961) ruled out the possibility that the observed incorporation of selenite was due to adaptation, adsorption or deposition of elemental selenium. This could hold true only for very low selenite concentrations as the results obtained here indicate; furthermore, it was shown by Nickerson and Falcone (1963) that selenium could become chemically bound to thiol groups of proteins.

2. Non-proliferating cells

a. Effect of pH and selenite concentration on selenite reduction and uptake In order to elucidate possible differences in the action of selenite on the three selected organisms, a number of experiments were performed using resting cell suspensions which had been adjusted to contain 10 mg/ml, as described under Materials and Methods. The net uptake is shown in Figures 10 to 12. Total selenite utilization, net uptake and reduction had their optima at pH 7.0. Net uptake was again lowest for P. vulgaris whereas the maximum reduction by E. coli was considerably lower than the peaks obtained with P. vulgaris and S. thompson. One could conclude from this that on a dry weight basis P. vulgaris and S. thompson possessed a stronger reducing power than E. coli, and the reduction of selenite was a defense mechanism of the organism against the toxic effect of the selenium salts.

Increasing selenite concentrations had almost identical effects on the total uptake of selenite by all three microorganisms. Over a concentration range from 10 μg selenite/ml to 80,000 μg/ml there was an increased total uptake of selenite up to 20,000 μg/ml with a plateau between 250 to 1,000 μg. From 20,000 to 80,000 μg selenite/ml, the uptake

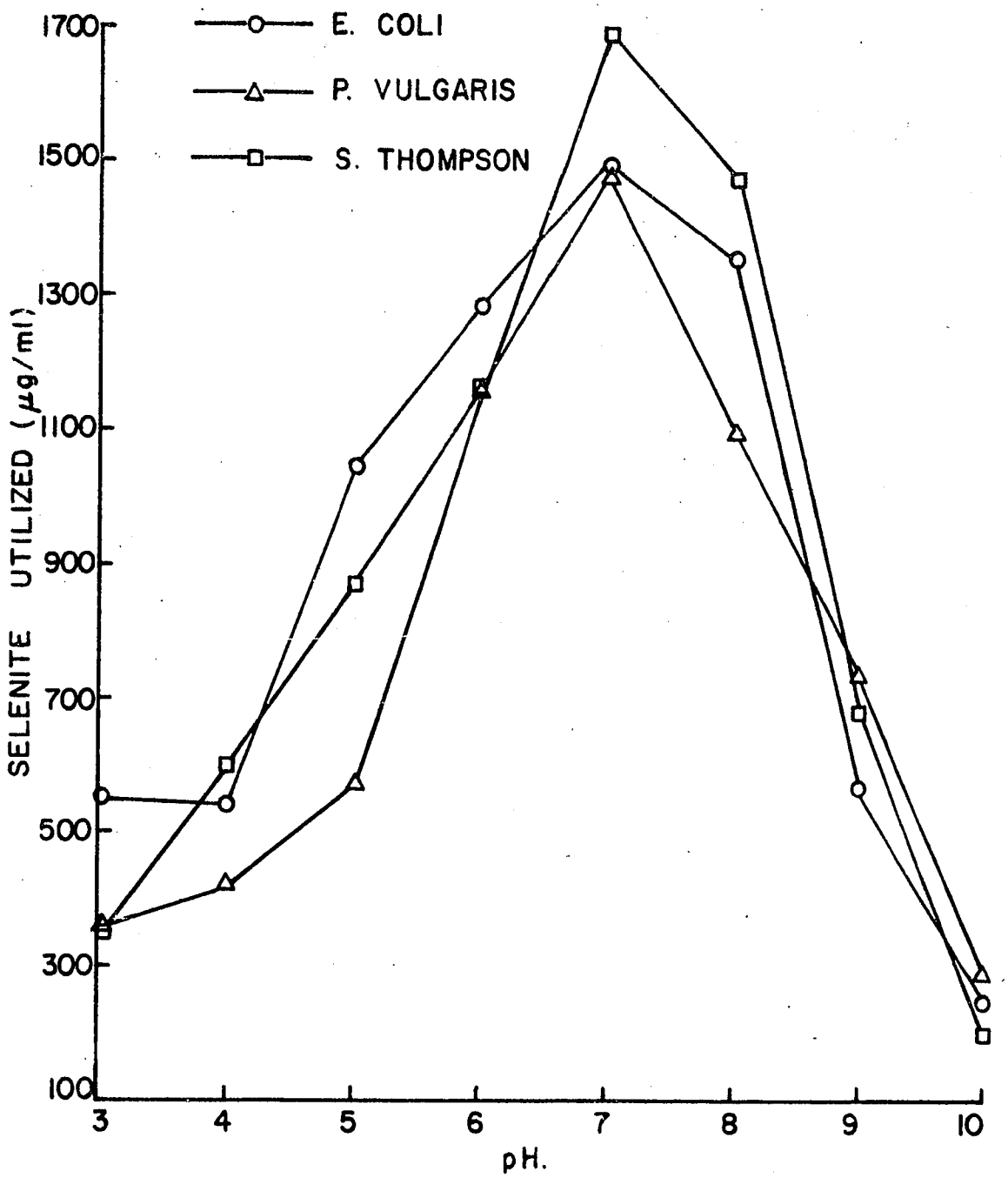


Figure 10. Effect of pH on total utilization of selenite by resting cells

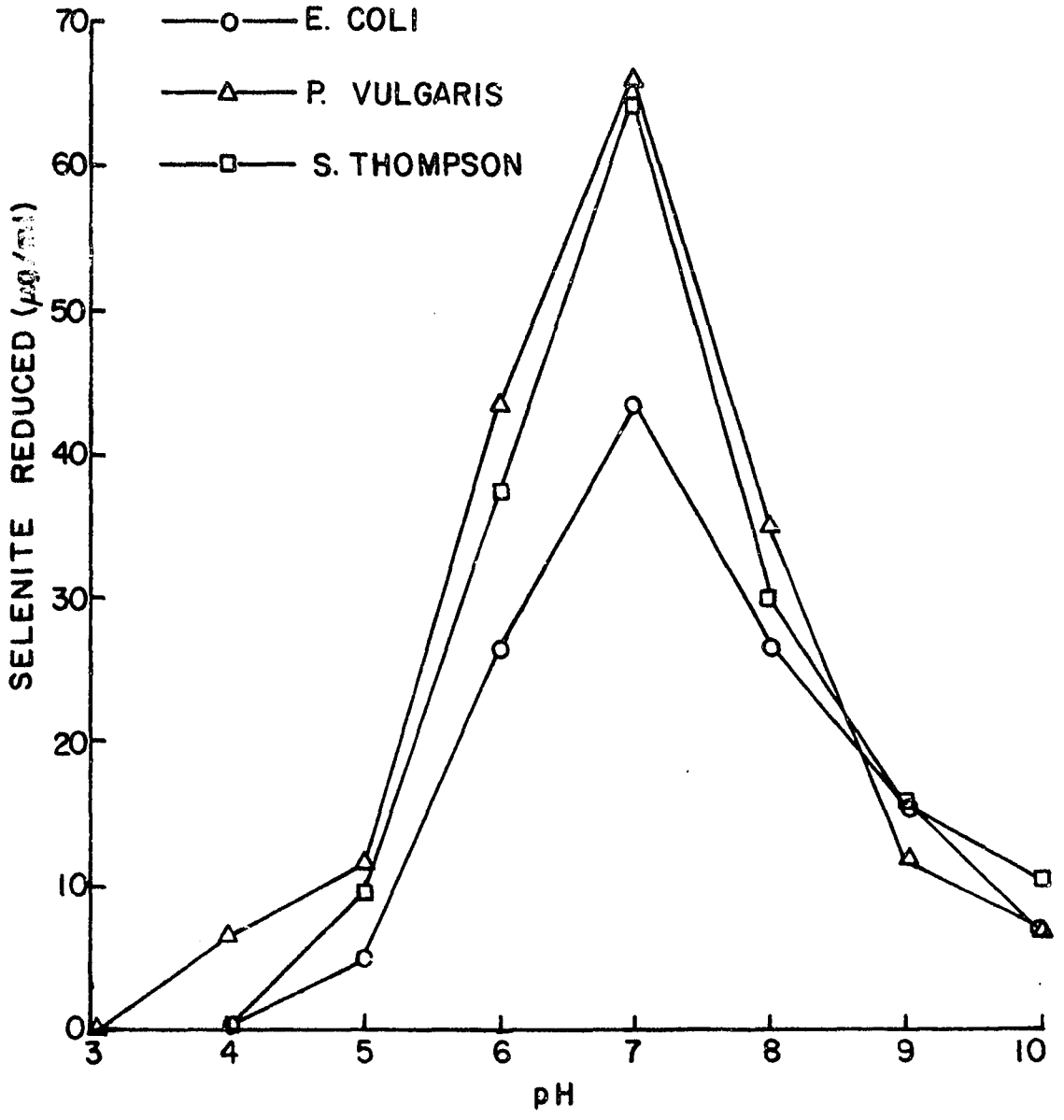


Figure 11. Effect of pH on reduction of selenite

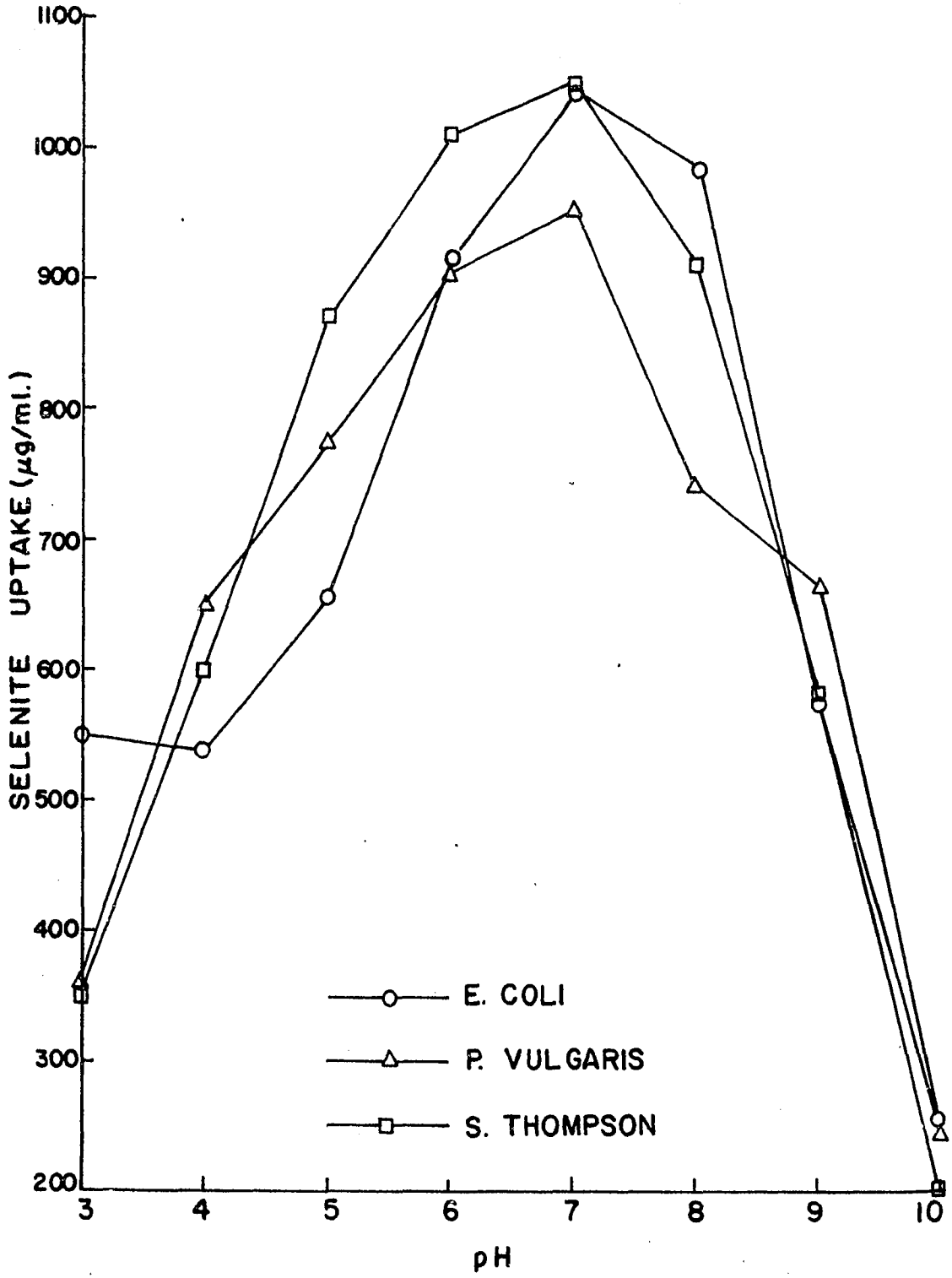


Figure 12. Effect of pH on net-uptake of selenite

remained fairly constant (Table 2).

The effect of increasing selenite concentration on reduction was entirely different from that obtained for selenite uptake. The reduction curve for each of the three organisms (Figure 13) showed a definite peak, but differences in the effect of increasing selenite concentrations are indicated by the fact that the optimum selenite concentration to obtain maximum reduction for E. coli was approximately one half of that required for S. thompson and P. vulgaris.

b. Effect of inorganic ions on selenite uptake and reduction It was shown above that added inorganic ions influenced the toxicity of selenite toward growing cells of the three test organisms. The following experiments were conducted to show the effect of various concentrations of phosphate, sulfate and sulfite on the utilization of selenite by the three organisms on a dry weight basis.

The results (Figure 14) showed that the utilization of selenite was depressed by the three ions at all concentrations tested. If the toxic action of selenite were a direct function of the amount taken up by the bacterial cells, one would expect a substance that decreases selenite uptake to lessen its toxicity. Disappearance of selenite from the

Table 2. Effect of selenite concentration on total uptake of selenite by resting cell suspensions

Selenite Concentration (μg/ml)	Selenite uptake *E. coli* %	*E. coli* (μg/ml)	*P. vulgaris* %	*P. vulgaris* (μg/ml)	*S. thompson* %	*S. thompson* (μg/ml)
10	86.5	8.7	81.0	8.1	85.2	8.5
100	75.0	75	74.0	74	72.5	72.5
250	70.0	175	64.5	170	64.8	162
500	35.0	175	40.0	200	42.5	215
1000	15.5	155	16.8	168	17.8	178
5000	12.0	600	13.2	600	14.2	710
10000	10.5	1000	9.5	950	10.4	1040
20000	8.0	1600	7.5	1500	8.0	1600
40000	3.5	1400	4.0	1600	3.7	1480
80000	2.0	1600	2.3	1800	1.9	1520

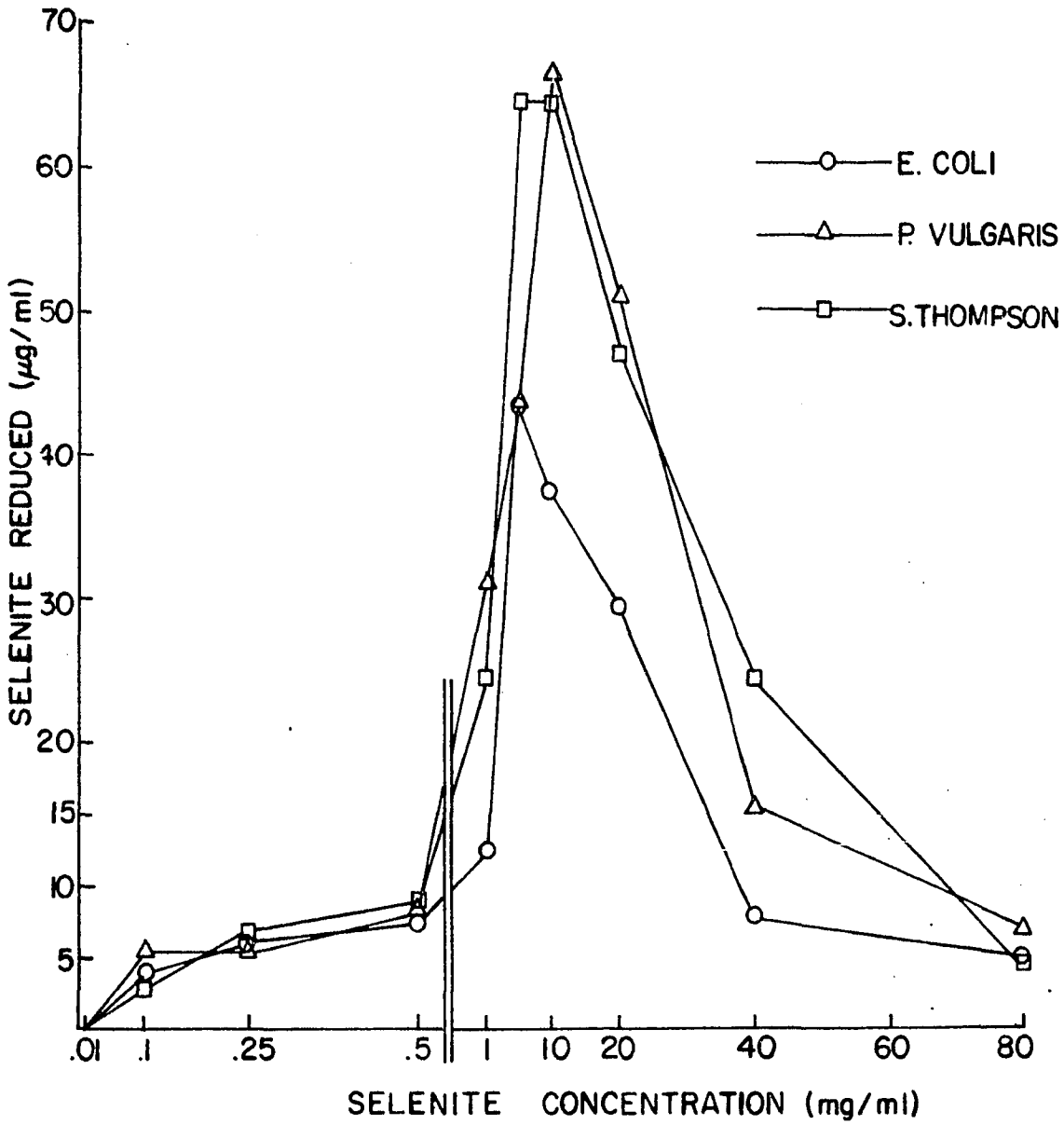
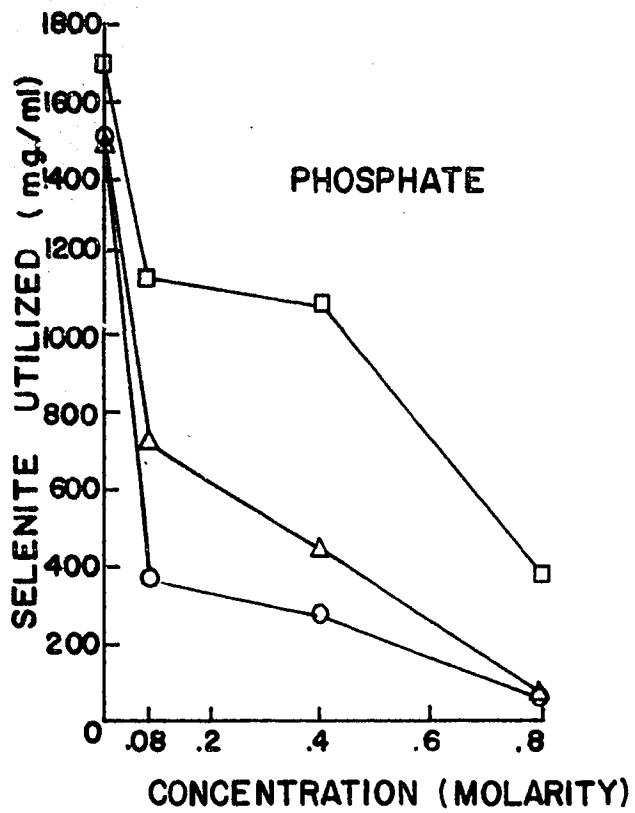
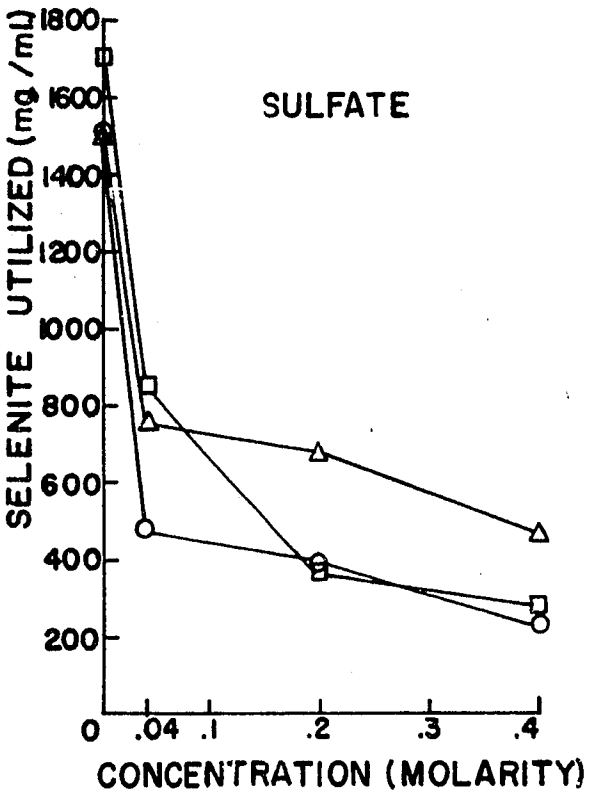
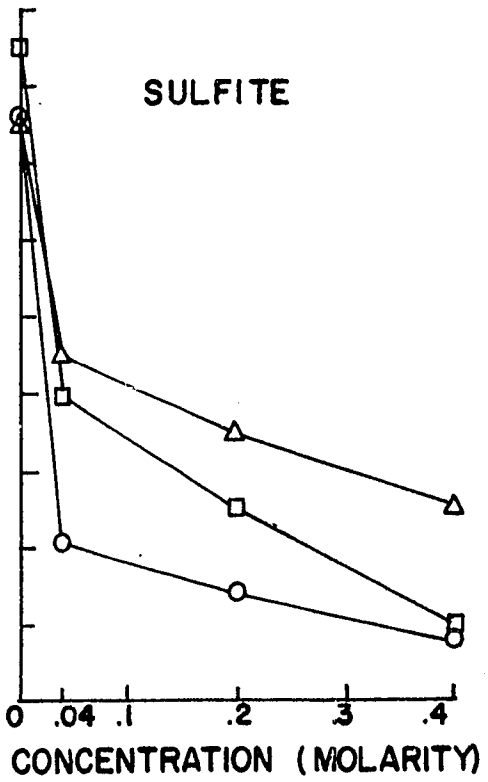


Figure 13. Effect of selenite concentration on reduction of selenite

Figure 14. Effect of inorganic ions on utilization of selenite by E. coli, P. vulgaris and S. thompson


PHOSPHATE
SELENITE UTILIZED (mg./ml)
1800
1600
1400
1200
1000
800
600
400
200
0
.08
.2
.4
.6
.8
CONCENTRATION (MOLARITY)
E. COLI
P. VULGARIS
S. THOMPSON


SULFATE
SELENITE UTILIZED (mg./ml)
1800
1600
1400
1200
1000
800
600
400
200
0
.04
.1
.2
.3
.4
CONCENTRATION (MOLARITY)


SULFITE
0
.04
.1
.2
.3
.4
CONCENTRATION (MOLARITY)

supernatant was measured, however, rather than uptake per se by the cells. This meant that incorporation of selenite into the water space* of the cells (non-metabolic uptake) was included in "uptake", and competition between selenite and the added ions was at least, in part, for the water space rather than the solid cell material.

Figure 14 shows that there were quantitative differences in the inhibition of selenite uptake between the three different microorganisms for each added ionic species; however, no meaningful relationship appeared to exist between the results obtained with the growing organisms and the data for the resting cell suspensions. It was possible, though, that at a lower total salt concentration, phosphate would have lessened the toxicity of selenite toward growing organisms by suppressing metabolic uptake of selenite, if indeed the action of phosphate was on the metabolic and not on the water space uptake of the cells.

The action of the sulfur salts on resting cells may be identical to that of phosphate; however, with growing

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*Water space of the cell is defined as that part of the total volume of a cell which has the same concentration of diffusible material as that found in the medium (Roberts et al., 1955).

organisms one would expect a sparing action, that is, a direct competition with selenite for incorporation into the cells via the sulfur metabolic pathways. Under certain conditions, inorganic sulfur salts may increase selenite toxicity by an action similar to that mentioned for methionine, by retaining a greater concentration of $SeO_3^=$ in the medium.

3. Distribution of radio-selenium among cell fractions in the different bacterial species

a. Non-protein fractions Studies of the sulfur metabolism in E. coli by Roberts et al. (1955) showed that this element was not found exclusively in the residual protein fraction of the cell. According to Roberts et al., the cold-TCA soluble fraction probably contained most of the inorganic ions, but of particular interest was the relatively high abundance of glutathione. The authors pointed out, however, that the distribution of tracers among the fractions depended upon the length of incubation time, the composition of the medium, and the general growth conditions. A typical distribution of $S^{35}O_4^=$ in the five fractions of E. coli as given by Roberts et al. was as follows: cold-TCA soluble, 23.6%; alcohol soluble, 13.7%; alcohol-ether soluble, 0.54%;

hot-TCA soluble, 2.05%; residual protein, 56.1%. The distribution of Se^{75} among the fractions of the three organisms is given here for comparative purposes only, and no correlation to the distribution of S^{35} is sought. As can be seen from the data in Table 3, under the conditions of the experiment there were no marked differences in the distribution of Se^{75} among the fractions of the three bacterial species.

b. Residual protein (formation of seleno analogues of sulfur-containing amino acids) 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. Ninety ml of 1.5N HCl in 5 ml portions were used first, followed by 30 ml of 6N HCl also in 5 ml fractions. The elution patterns are presented in Figure 15. E. coli showed one rather sharply defined peak between 5 and 25 ml of 1.5N HCl and one between 5 and 20 ml of 6N HCl. From P. vulgaris, only a few fairly active fractions were obtained with 1.5N 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 gradual decrease in activity indicated the presence of compounds having more diverse Dowex 50 properties than in the case of E. coli.

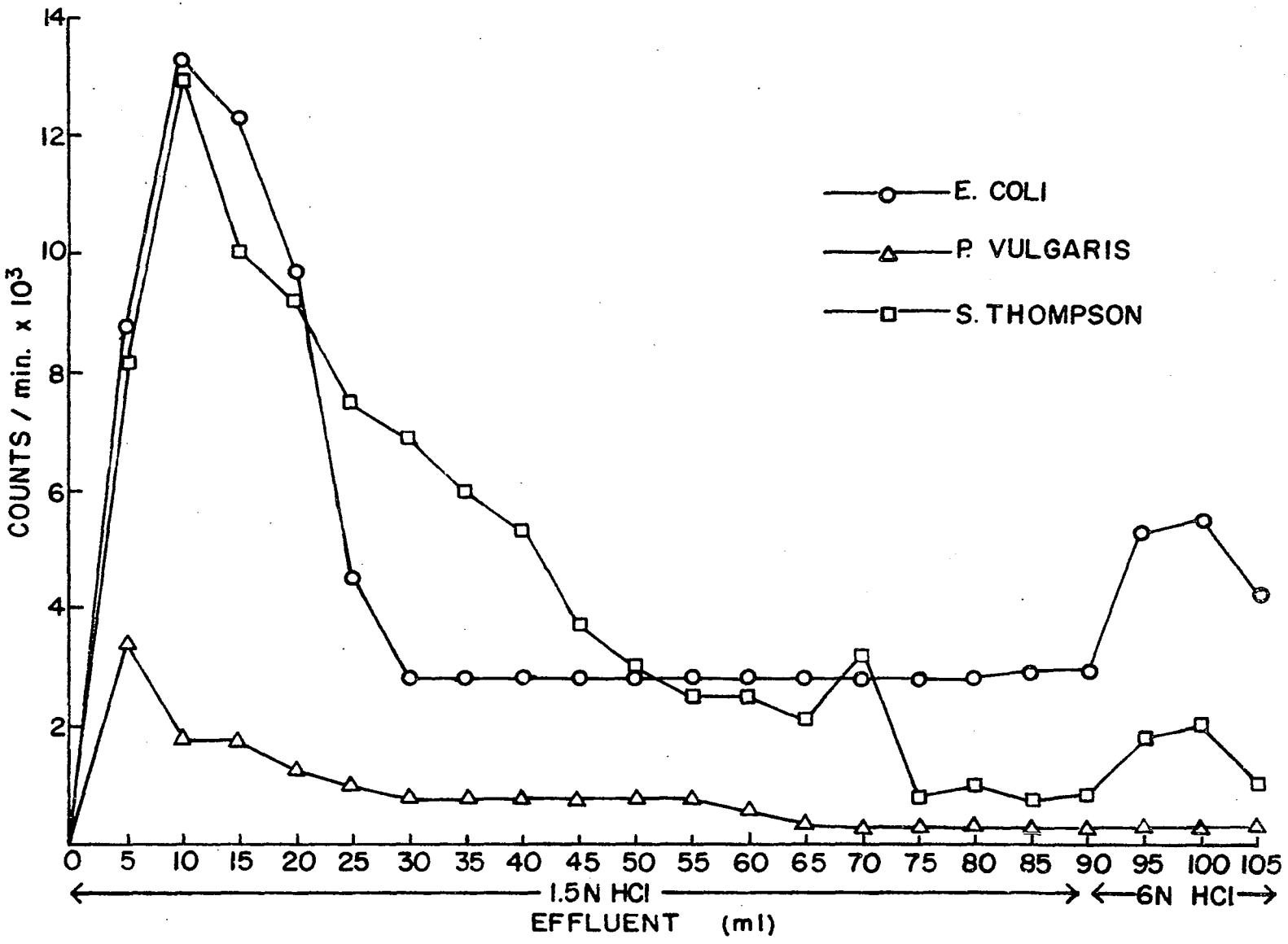
Table 3. Distribution of selenium in cell fractions

Fraction	Percentage range of radioactivity		
	E. coli	P. vulgaris	S. thompson
Washed cells	100	100	100
Cold-TCA-soluble	0.029- 0.080	0.026- 0.050	0.153- 0.190
Alcohol-soluble	0.290- 0.580	0.075- 0.175	0.224- 0.300
Alc.-ether-sol.	0.350- 0.600	0.260- 0.364	0.750- 0.833
Hot-TCA-soluble	0.023- 0.050	0.015- 0.027	0.029- 0.060
Residual protein	88.231-91.487	86.419-90.747	93.145-96.015
Total recovered	88.913-92.797	86.795-91.363	93.301-96.338

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

The four final solutions were chromatographed and the chromatograms were radioautographed to locate the position of

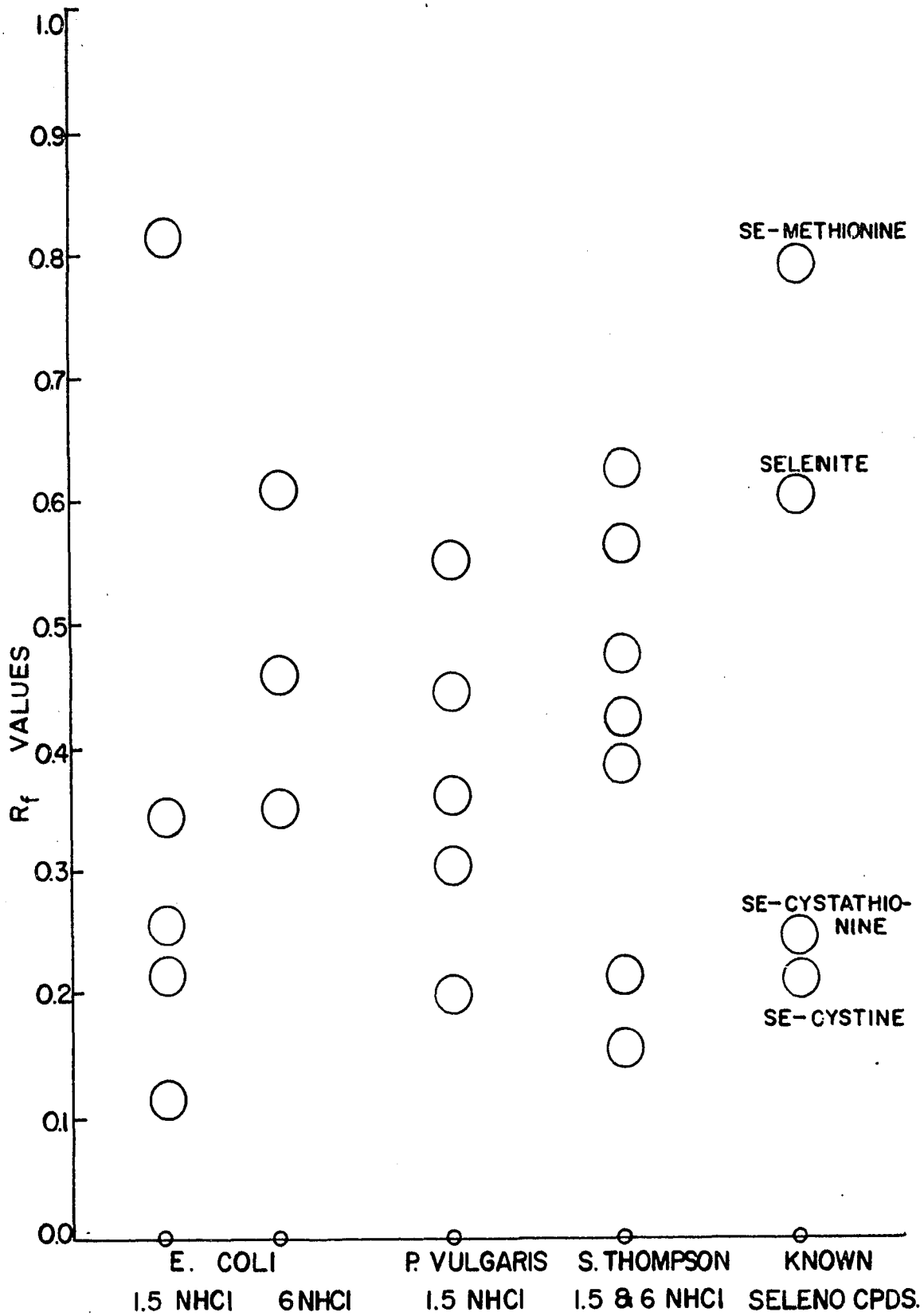
Figure 15. Column chromatography of protein hydrolysates of *E. coli*, *P. vulgaris* and *S. thompson*


14
12
10
8
6
4
2
COUNTS / min. x 10^3
E. COLI
P. VULGARIS
S. THOMPSON
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95 100 105
1.5N HCl
6N HCl
EFFLUENT (ml)

the radioactive material. Figure 16 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, and the "fingerprints" (see Materials and Methods) confirmed the identification. As could be 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. Seleno-cystine, but neither seleno-cystathionine (the second spot was "fingerprinted" but did not superimpose) 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-

Figure 16. Line drawing of radioautographs and chromatograms of HCl effluents of *E. coli*, *P. vulgaris*, and *S. thompson*


1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
R_f VALUES
SE-METHIONINE
SELENITE
SE-CYSTATHIO-NINE
SE-CYSTINE
E. COLI
1.5 NHCl
6 NHCl
P. VULGARIS
1.5 NHCl
S. THOMPSON
1.5 & 6 NHCl
KNOWN
SELENO CPDS.

compound. Tuve (1958), in his studies on E. coli, obtained a compound having an R_f value of 0.55 in the same solvent.

The 1.5N 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 6N HCl fractions, but the presence of selenite was proved suggesting that the selenite identified for S. thompson was present in the 6N HCl fractions of this organism.

From Figure 16 it may be observed that several of the unidentified compounds from the three organisms have similar R_f values, suggesting a similarity in the metabolic pattern. 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 cost of the toxic seleno-cystine could in part have accounted for the resistance to selenite exhibited by these two species. It may be assumed that some of the unidentified spots represented oxidation products of seleno-methionine. These spots, however, were present, also, on the radioautographs of E. coli, indicating that conversion per se of cystine to methionine was not a differentiating factor in selenite resistance between the three organisms. Kinetic aspects of selenite uptake, rather

than the ultimate localization of selenite in the cell protein, appear to be the factor that determines the degree of resistance or of susceptibility to selenite.

To make the selenite enrichment medium more selective in favor of Salmonellae, one would have to induce rapid selenite uptake by non-Salmonella organisms and delay uptake of selenite by Salmonellae.

IV. SUMMARY

Growing cells of E. coli, P. vulgaris and S. thompson showed striking differences in their tolerances to increasing concentrations of sodium selenite in tryptone phosphate broth. It was found that 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 3.00%. S. thompson was inhibited completely by 3.00% selenite but not by 2.50%, although there was a considerable lag and a decrease in total growth.

The effect of sulfate, sulfite, and phosphate on the toxicity of selenite for the three bacteria was tested by adding high concentrations of the salts to the basal medium at two different selenite levels. The toxic effect of selenite was not lessened for any of the three organisms by the addition of 10% phosphate. Five percent sulfate also generally enhanced the toxicity of selenite; P. vulgaris was most severely affected. Sulfite, at a concentration of 0.15%, markedly influenced the growth of S. thompson at the low selenite level. The results for E. coli and P. vulgaris were similar to those with added sulfate.

Neither added cystine nor methionine or histidine

reversed selenite toxicity for E. coli. Inhibition was probably completed before any sparing action could take effect. The growth of P. vulgaris was enhanced by all three amino acids. With S. thompson, cystine and histidine increased the final count, but methionine depressed growth considerably.

The relationship between growth, uptake and reduction of selenite was determined at two widely different levels of selenite. The same general pattern of total utilization and net uptake of selenite was obtained for both selenite concentrations.

The influence of pH and of selenite concentration on the uptake and reduction of selenite was investigated using non-proliferating cells, and the effect of the three inorganic ions was also studied further.

Optima for both net uptake and for reduction of selenite were at pH 7 for all three organisms. Net uptake was lowest for P. vulgaris, whereas E. coli had the smallest reduction maximum. The effect of increasing selenite concentration on apparent total uptake and on reduction of selenite was almost identical for the three organisms. Over a concentration range from 10 to 20,000 μg $SeO_3^{=}$/ml, there was an increased

total uptake. From 20,000 to 80,000 μg, uptake remained nearly constant. The reduction curves, however, showed definite peaks, but the optimum selenite concentration to obtain maximum reduction for E. coli was approximately one-half of that required for P. vulgaris and S. thompson.

All three inorganic ions depressed uptake of selenite by the three bacterial species, suggesting that the toxic action of selenite was not a direct function of the amount of selenite taken up by the bacterial cell.

The distribution of radio-selenium among the cell fractions in the different bacterial species was also determined. No significant differences in selenite uptake by the various fractions of the three microorganisms were observed.

Seleno-analogues of sulfur-containing amino acids were determined by column chromatography and radioautography. Radioautographs of the protein hydrolysates of the three bacterial species revealed the presence of seleno-cystine in all three organisms. The presence of seleno-methionine could be shown only for E. coli, but compounds having R_f values corresponding to possible oxidation products of seleno-methionine were present in the hydrolysates of P. vulgaris and S. thompson.

V. CONCLUSIONS

1. Selectivity for S. thompson is not increased by addition of high concentrations of phosphate, sulfate, or sulfite to a selenite tryptone phosphate broth, or by supplementation of the broth with cystine, methionine, or histidine.

2. Utilization of selenite by non-proliferating cells of E. coli, P. vulgaris, and S. thompson is decreased by addition of the three inorganic ions.

3. The ability of the three bacterial species to reduce selenite is influenced by the selenite concentration. High levels of selenite which permit maximum reduction by P. vulgaris and S. thompson depress reduction by E. coli.

4. The optimum pH value is 7 for net uptake and reduction of selenite by E. coli, P. vulgaris and S. thompson.

5. E. coli, which is more susceptible to selenite than are P. vulgaris and S. thompson, incorporates a greater amount of selenium than do the other two organisms (μg selenium/mg dry weight of cells) during the early stages of incubation.

6. In a selenite enrichment medium, the qualitative distribution of selenium in the cell protein as seleno-analogues of sulfur-containing amino acids does not differ

appreciably between E. coli, P. vulgaris, and S. thompson.

7. The selectively inhibitory action of sodium selenite on E. coli, P. vulgaris, and S. thompson is primarily a function of the rate of selenite uptake, whatever the ultimate fate of the selenium ion may be in the bacterial cell.

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