Anion Antagonisms in Yeast as Indicators of the Mechanism of Selenium Toxicity

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The uptake by plants of toxic quantities of selenium presents a serious problem to the livestock industry in numerous semiarid regions. The antagonism between selenium and arsenic presented an opening for a study of selenium metabolism in yeast. Yeast respiration is strongly inhibited by $10^{-4}M$ selenite when glucose or ethyl alcohol is used as substrate but not when lactate, pyruvate, or acetate is used. Inhibition of yeast respiration by selenite is reduced in the presence of arsenite, arsenate, or phosphate. Selenate inhibition is only partially reduced by a combination of arsenate and phosphate. Selenite or arsenate inhibition is reduced by phosphate, but selenate or arsenite inhibition is not affected.

THE ANTAGONISTIC RELATIONSHIP between selenium and arsenic, first reported by Moxon in 1938 (*11*), has been the subject of numerous papers (*7–9*), most of which have originated from this laboratory. This antagonism is of paramount interest in the study of the mechanism of selenium toxicity because it supplies evidence concerning one step in the metabolism of ingested selenium. When fed to rats, arsenic seems to be equally effective against many forms of selenium (*4*), but when the selenium is injected the antagonism has been demonstrated only in the case of selenite (*3, 7*). While the reasoning is not definitive, this may be taken as presumptive evidence that orally ingested selenium is converted to selenite before exerting its toxic effect.

The large number of animals required for injection studies led to a consideration of the use of yeast as a test organism. Lardy and Moxon (*10*) reported that $6.25 \times 10^{-4}M$ selenite inhibited carbon dioxide production of fermenting yeast about 50% and that the addition of $1.33 \times 10^{-4}M$ arsenite reduced the inhibition to about 30%. Potter and Elvehjem (*13*) and Berry and Pengra (*2*) investigated the effect of selenium on respiration of yeast. The latter workers reported that arsenic was not antagonistic under the conditions of their experiment. The selenium concentrations used by these two groups of workers were so high ($3.33 \times 10^{-3}M$ and above) that it seemed probable that further studies would be of no value unless an interfering factor or factors could be removed by changing the conditions under which the inhibition was studied. Accordingly, a survey was initiated, some of the results of which are reported here. As the influence of some of the conditions is not yet defined, only those results that seem most significant are included. The material is presented in graphic form, for the general trends are readily reproducible over a considerable range of conditions while exact values are not.

Because arsenic and phosphorus follow somewhat similar metabolic routes, the phosphate buffer was replaced by glycine. In an attempt to reduce the ease with which selenite would oxidize unstable compounds, the studies were carried out at pH 3. In accordance with the findings of Rothstein and Demis (*14*), potassium chloride was added to minimize the toxic effect of the hydrogen ion. A stock solution of buffer was prepared by dissolving 15 grams of glycine, 3 grams of potassium chloride, and 2.5 ml. of concentrated hydrochloric acid in water and diluting to 500 ml. Commercial cake yeast (Fleischmann) was suspended in water and washed twice, and 2 ml. of the wet yeast was drawn into a pipet without disturbing the starch cake at the bottom of the centrifuge tube. This wet yeast was suspended in 67 ml. of water and aerated by shaking overnight. A conventional Warburg apparatus with the temperature set at 31° C. was used for the studies of yeast respiration. Each flask contained, in a liquid volume of 3 ml., 1.5 ml. of buffer and 0.5 ml. of the yeast suspension (ca. 15 mg. of wet yeast). Unless specifically described, the substrate consisted of 50 mg. of glucose per flask. In all cases substrate was still present in excess at the end of 4 hours. For simplicity of operation, reagents were made up to six times the desired concentration in either water or buffer and the final volume in the flask was made up of the proper combination of 0.5-ml. portions. The pH of salts and acids was adjusted to approximately that of the buffer. Addition and equilibration were timed in such a way that the yeast was added 30 minutes before the stopcocks were closed. In most cases, the selenium salt was placed in the side arm and dumped at zero time.

Potter and Elvehjem (*13*), using $3.33 \times 10^{-3}M$ selenite, concluded that the toxic effect was exerted chiefly on glycolysis. With this as a basis and to establish a reference point from which to proceed, a series of runs was made using 2.5×10^{-3} and $1.25 \times 10^{-3}M$ selenite. For each flask set up to record oxygen uptake—i.e., with potassium hydroxide in the center well—there was a duplicate flask with water in the center well to indicate the volume of carbon dioxide given off in excess of the oxygen used. This excess or fermentation carbon dioxide added to the volume of oxygen used in the duplicate flask then gave a measure of the total carbon dioxide produced by the yeast under a given set of conditions. The results of several of these runs were averaged and are plotted in Figure 1. Under these conditions, the total carbon dioxide production was almost identical for the selenized and the control flasks, the apparent absence of the Pasteur effect in the flasks containing selenium almost exactly compensating for the decrease in oxygen uptake. In other words, glucose

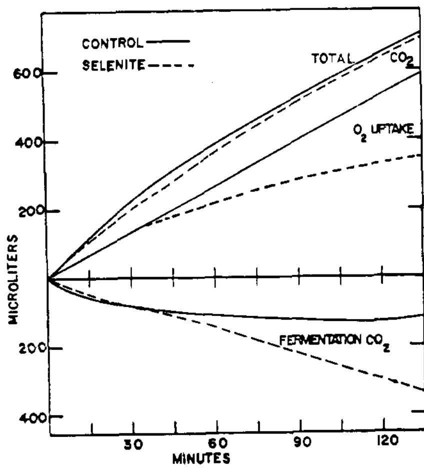


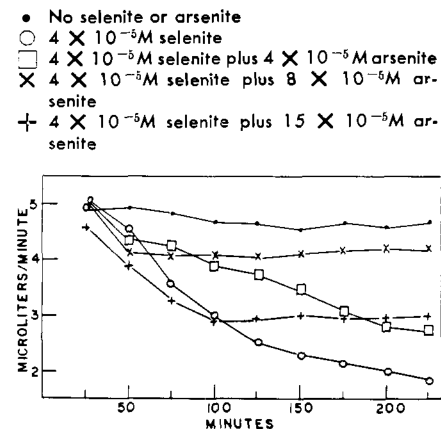
Figure 1. Oxygen uptake, fermentation, and total carbon dioxide produced yeast in presence and absence of selenite

breakdown proceeded at a more rapid rate in the presence of selenium, as because of the less efficient metabolism, the yeast in the flask containing selenium requires more glucose to produce the same amount of carbon dioxide.

After the first few runs it became apparent that a considerable period elapsed before the oxygen uptake of the selenized yeast approached constant rate. While the continued decline in rate of uptake may be due to secondary effects, this is not a serious objection to the use of a longer time, if the uptake shows reproducible differences with varying quantities of selenite. Rats injected with lethal doses of selenite survive for several hours, so the comparable process in yeast cannot be expected to produce maximal inhibition at once. The data obtained from preliminary runs indicated that the oxygen uptake became reasonably constant about 200 minutes after addition of selenite to the actively respiring yeast. The inhibition produced by various levels of selenite is shown in Figure 2 and the corresponding values for selenate are shown in Figure 3.

Figure 4. Arsenite reversal of inhibition produced by selenite

- • No selenite or arsenite
- ○ $4 \times 10^{-5}M$ selenite
- □ $4 \times 10^{-5}M$ selenite plus $4 \times 10^{-5}M$ arsenite
- × $4 \times 10^{-5}M$ selenite plus $8 \times 10^{-5}M$ arsenite
- + $4 \times 10^{-5}M$ selenite plus $15 \times 10^{-5}M$ arsenite



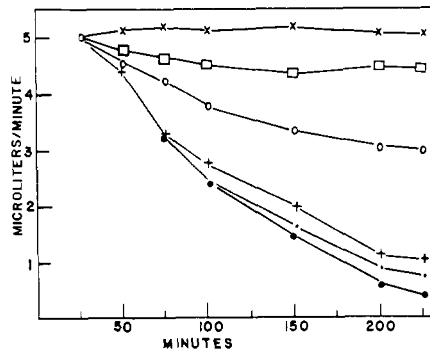


Figure 2. Inhibition of yeast respiration by various levels of selenite

- × No selenite
- □ $1.25 \times 10^{-5}M$ selenite
- ○ $2.5 \times 10^{-5}M$ selenite
- + $5 \times 10^{-5}M$ selenite
- • $12.5 \times 10^{-5}M$ selenite
- ● $250 \times 10^{-5}M$ selenite

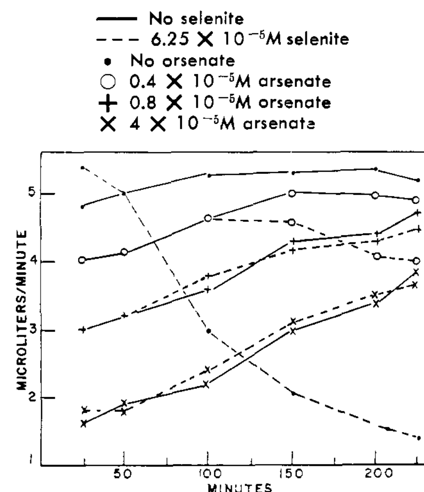
The shapes of the curves vary little as the concentration of selenite in the flasks is reduced to $12.5 \times 10^{-5}M$, while further reduction permits an increase in uptake to a value near that of the controls. The presence of an almost identical lag period at all concentrations is evidence that a chemical reaction, the rate of which is determined by the metabolic state of the organism, must take place before inhibition occurs.

The selenite concentration for the selenite-arsenite antagonism studies was selected to give an oxygen uptake about one third that of the control, so that either antagonism or an additive effect could be detected. As is shown in Figure 4, arsenite will almost completely reverse the inhibition produced by selenite if concentrations are carefully adjusted. As the lowest effective concentration of arsenite is somewhat toxic, the value of arsenite in mechanism studies such as these is questionable. Arsenate had been reported to be relatively nontoxic to yeast, so a few runs were made to check its effectiveness in reversing the effect of selenite. Arsenate proved to be antagonistic to selenite at much lower levels than arsenite but, under the conditions of this experiment, it is so toxic that the net result is almost the same. These results are plotted in Figure 5.

The surprisingly high toxicity of arsenate made it seem advisable to determine the toxicity of selenite, selenate, arsenite, and arsenate in the presence of phosphate. The results as plotted in Figure 6 show that phosphate almost completely masks the effect of selenite and arsenate, does not alter the effect of selenate, and increases somewhat the toxicity of arsenite. These results point up the differences in the physiological action of the four ions. In the case of selenate and selenite, this may be a difference of degree, but arsenite is set

Figure 5. Arsenate reversal of inhibition produced by selenite

- —— No selenite
- - - - $6.25 \times 10^{-5}M$ selenite
- • No arsenate
- ○ $0.4 \times 10^{-5}M$ arsenate
- + $0.8 \times 10^{-5}M$ arsenate
- × $4 \times 10^{-5}M$ arsenate



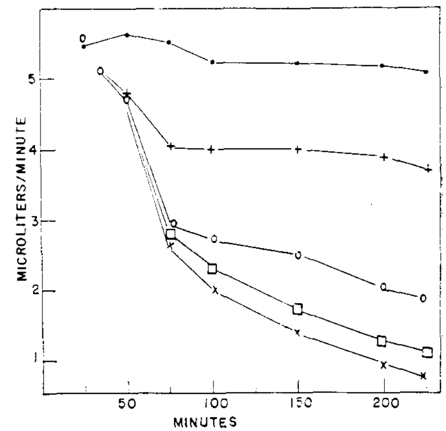
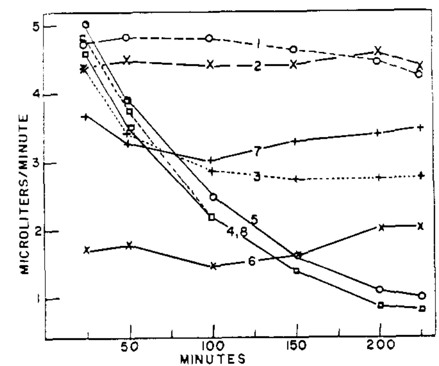


Figure 3. Inhibition of yeast respiration by various levels of selenate

- • No selenate
- + $0.4 \times 10^{-5}M$ selenate
- ○ $0.8 \times 10^{-5}M$ selenate
- □ $1.6 \times 10^{-5}M$ selenate
- × $25 \times 10^{-5}M$ selenate

Figure 6. Influence of phosphate on inhibition of yeast respiration by four ions

1 through 4. - - - 5×10^{-4} M phosphate
5 through 8. —— No phosphate
1, 5. $6.25 \times 10^{-5}M$ selenite
2, 6. $6.25 \times 10^{-5}M$ arsenate
3, 7. $12.5 \times 10^{-5}M$ arsenite
4, 8. $6.25 \times 10^{-5}M$ selenate



apart from the others by a complete independence from any interference by phosphate.

The effect of phosphate on selenite inhibition was studied thoroughly because it promised to yield information on the mechanism of the reaction in which the members of this group of ions appear

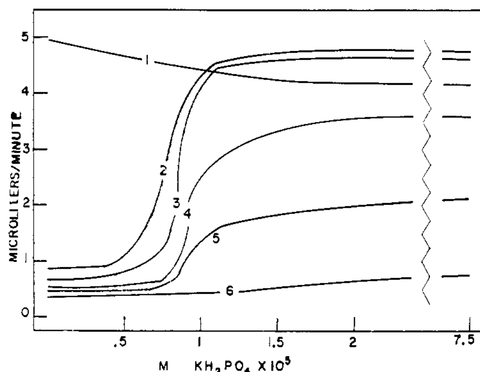


Figure 7. Oxygen uptake

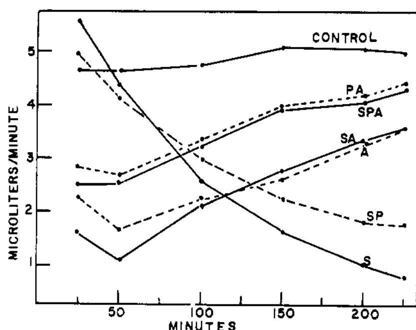
200 minutes after addition of various levels of selenite to respiring yeast suspensions containing added phosphate

1. No selenite
2. 6.25 $\times$ 10^{-5}M selenite
3. 12.5 $\times$ 10^{-5}M selenite
4. 25 $\times$ 10^{-5}M selenite
5. 50 $\times$ 10^{-5}M selenite
6. 250 $\times$ 10^{-5}M selenite

to compete. Phosphate was placed in the flasks and selenite dumped from the side arms at zero time. While the oxygen uptakes were usually recorded over a considerable period, those for the period from 200 to 225 minutes were selected for comparison. In Figure 7 oxygen uptakes per minute for this period are plotted against phosphate concentrations for various levels of selenite. One rather puzzling phenomenon appeared at the two highest levels of selenite; the flasks without phosphate always contained easily visible quantities of red metallic selenium. This red color was much less evident in

Figure 8. Inhibition of yeast respiration by selenite in presence of arsenate and phosphate

S. 12.5 $\times$ 10^{-5}M selenite
P. 10 $\times$ 10^{-5}M phosphate
A. 4 $\times$ 10^{-5}M arsenate



the phosphate flasks, even those containing so little phosphate that it had no effect on oxygen uptake.

As both arsenate and phosphate reverse selenite toxicity, it could be predicted that they would do so in combination. An assortment of curves may be obtained by varying the three constituents. Figure 8 shows that at these levels of phosphate, selenite, and arsenate the selenite has no effect on respiration in the presence of arsenate. These curves also illustrate the rather dubious value of percentage inhibitions calculated for any given length of time. Depending on the length of time after the addition of the two salts, arsenate enhances, has no effect on, or diminishes the inhibition produced by selenite.

The values plotted in Figure 9 were selected because they show that selenate, although much more refractory than selenite, does yield to treatment with arsenate plus phosphate.

Because the survey to this point had led to the conclusion that these ions

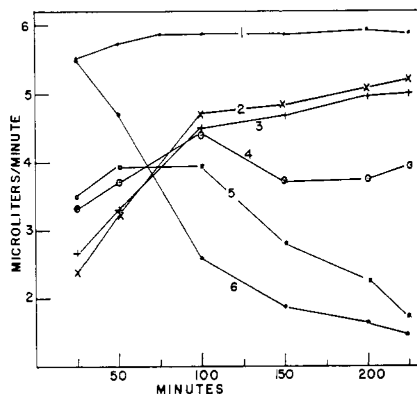


Figure 9. Inhibition of yeast respiration by selenite and selenate in presence of arsenate and phosphate

1. No selenate, arsenate, or phosphate
2. 8 $\times$ 10^{-5}M arsenate, 4 $\times$ 10^{-4}M phosphate
3. 8 $\times$ 10^{-5}M arsenate, 4 $\times$ 10^{-4}M phosphate, 10 $\times$ 10^{-5}M selenite
4. 8 $\times$ 10^{-5}M arsenate, 4 $\times$ 10^{-4}M phosphate, 1 $\times$ 10^{-5}M selenate
5. 8 $\times$ 10^{-5}M arsenate, 4 $\times$ 10^{-4}M phosphate, 5 $\times$ 10^{-5}M selenate
6. 1 $\times$ 10^{-5}M selenate

were competing in a reaction or a group of closely related reactions, it seemed of interest to try to determine the nature of the organic compound or compounds involved. Wright (*17*) reported that pyruvate oxidation by liver slices is not inhibited by selenium, if the pyruvate is added to the slices before or with the selenite, but that addition of pyruvate will not restore the uptake of poisoned slices. It has also been reported (*13*) that selenite inhibits only slightly the oxidation of pyruvate and lactate by yeast. These results coupled with the fact that selenite does not inhibit glycolysis were interpreted as meaning that selenite did not inhibit the first reaction in which it was involved. This interpretation was tested by the use of various mixtures of glucose and lactate as substrates. The results (Figure 10) show that, while lactate oxidation is not affected by selenite in the absence of other substrate, the addition of a very small quantity of glucose promotes the formation of a material that inhibits lactate oxidation just as much as it inhibits glucose oxidation.

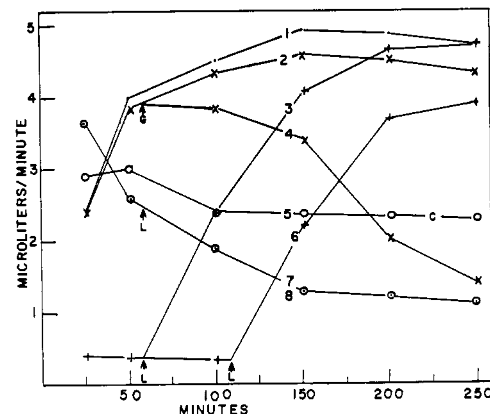


Figure 10. Inhibition of yeast lactate oxidation in presence of glucose

1. No selenite, 50 mg. lactic acid
2. 1 $\times$ 10^{-4} selenite, 50 mg. lactic acid
3. 1 $\times$ 10^{-4} selenite, 25 mg. lactic acid added at *L*
4. 1 $\times$ 10^{-4} selenite, 50 mg. lactic acid, 1 mg. glucose added at G
5. No selenite, 1 mg. glucose
6. 1 $\times$ 10^{-4}M selenite, 25 mg. lactic acid added at *L*
7. 1 $\times$ 10^{-4}M selenite, 25 mg. glucose
8. 1 $\times$ 10^{-4}M selenite, 25 mg. glucose, 25 mg. lactic acid added at *L*

Attempts to study the same system using ethyl alcohol in place of glucose were generally unsuccessful. When the concentrations of ethyl alcohol and lactate were essentially equal, the results were about the same as those obtained with glucose. On the other hand, when the concentration of ethyl alcohol was decreased results were very erratic, varying from virtually no inhibition one day to values approaching those of the glucose controls the next, even though concentrations and conditions were apparently the same. Results from several runs were averaged and are plotted in Figure 11.

Acetate and pyruvate are toxic to yeast at pH 3 and glycine has little buffer capacity near neutrality, so a citrate buffer with a pH of 6.5 was used for the study of these substrates. In the absence of glucose, there was no inhibition of acetate oxidation and only about 10% inhibition of pyruvate oxidation. The final level of uptake by yeast that had been incubated with glucose and

selenite was the same when acetate, lactate, or pyruvate was the substrate present in excess.

The results of this work agree well enough with those obtained by Wright (*17*) in his work with tissue slices to indicate that the mechanism is essentially the same in both cases. This is most easily seen in the time lag before inhibition appears and in the lack of inhibition of pyruvate oxidation. Supporting evidence for the similarity between the effects of selenite on yeast and mammalian tissue is the reversal of selenite toxicity in yeast by arsenite. A major difference is the fact that Wright found selenite to be more toxic than selenate to slices of all tissues that he used, while the reverse is true in yeast under the conditions of these experiments. Considering the wide variation in the effect of these salts on various tissues, this is not surprising. Also, if the original assumption, that all orally ingested selenium is converted to selenite before attacking the individual cell, is valid, the point becomes a minor one in the study of selenium poisoning, although interesting per se.

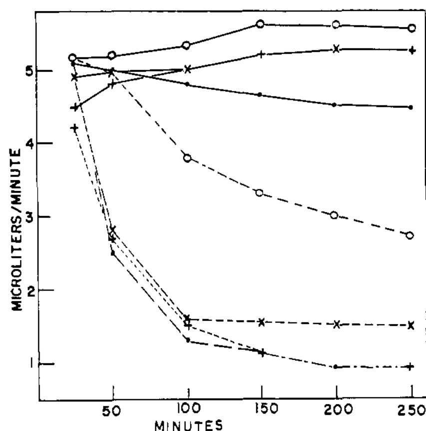


Figure 11. Inhibition of yeast lactate oxidation by selenite in presence of ethyl alcohol

—— No selenite
--- 1 × $10^{-4}M$ selenite

	Lactic Acid, Mg.	Ethyl Alcohol, Mg.
●	0	50
+	2	50
×	25	25
○	50	2

Wright also found that increasing the concentration of selenite reduces the period between addition of the salt and the appearance of inhibition in slices. Although this differs from the author's findings with yeast, it does not necessarily point to a difference in mechanism. It seems likely that the controlling factor in the case of yeast is the rate at which selenite enters the cell. It would not be surprising to find this control more complete in the undamaged cell than in the tissue slice. Attempts to study the comparable phenomenon in the whole animal are complicated by the fact that selenite attacks different systems at different rates and to a varying degree.

One indication of the complex nature of this problem may be obtained from the results of work on the storage of selenium in animal tissues (*8*). No difference was found in the quantity of selenium deposited in the tissues of rats when the symptoms of selenium poisoning were alleviated by the administration of arsenic. The stored selenium must have little toxic effect and must be metabolized by a route unaffected by the presence of arsenic.

While there is still little indication as to the mechanism by which selenium inhibits respiration, a few basic assumptions may be made. The present work indicates that selenite stimulates sugar breakdown in yeast, probably by attack on a controlling mechanism, and Wright found the same to be true in tissue slices. The first step could be looked upon as the formation of an ester with one of a number of anions as the acid and glucose or one of its metabolic products. The various antagonisms would then be the result of competition between the anions in the formation of esters. Gourley (*6*) and Sacks (*15*) as well as other workers have presented evidence that glycolysis is essential for phosphate transfer across cell membranes and that phosphate enters the cell in an organic form. With this as a point of departure, it is possible to develop a hypothesis to explain some of the results reported in this paper.

A reaction which can be blocked by arsenate or phosphate is essential for the conversion of selenite to a toxic material. The results obtained with phosphate and selenite indicate that the relationship between these two ions is not simple competition. Possibly a certain number of reactive sites are available to both ions and the first step in the conversion of selenite to a toxic material is the occupation by selenite of some of these sites. At higher selenite levels a second route, with which phosphate does not interfere, becomes more important.

The metabolic reaction involved must be a part of glycolysis, as pyruvate, lactate, or acetate will not promote the inhibition of respiratory activity by selenite. As arsenate is known to interfere with the uptake of phosphorus in the dehydrogenation of phosphoglyceraldehyde, it could be assumed that this is the reaction involved. Phosphoglyceraldehyde dehydrogenase will catalyze the uptake of inorganic phosphate with acetaldehyde as the oxidizable substrate. This would explain the promotion of selenite inhibition by ethyl alcohol.

The work of Bergstermann (*1*) assumes a certain importance at this point. He found that inhibition of phosphoglyceraldehyde dehydrogenase by selenite could be reduced by adding a hot-water extract of yeast. He assumed that the protection was due to cozymase in the extract. This extract could also have been a good source of phosphate.

The position of selenate and arsenite is not clear. Selenate behaves about the same as selenite when lactate and pyruvate are oxidized, and arsenite prevents selenite inhibition to a degree. It appears likely that these ions take part to some extent in the reaction in which the other three compete.

A further complication is the selenate-sulfate antagonism which has been studied by others (*5*, *12*, *16*). Of interest is the fact that sulfate prevents selenate uptake by *Chlorella* (*16*).

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