Metabolic Interactions of Selenate, Sulfate, and Phosphate

CARL W. BONHORST[1] AND IVAN S. PALMER[2]

Station Biochemistry, South Dakota Agricultural Experiment Station, College Station, Brookings, S. D.

Control of selenium uptake by plants shows promise as a practical approach to a solution of the selenium problem. As yeast selenite uptake is restricted by phosphate and plant selenate uptake by sulfate, the metabolic interactions of phosphate, sulfate, and selenate were studied in an effort to learn more about the restricting mechanism and to find a link between selenate and selenite metabolism. Selenate and sulfate both depress oxygen uptake by washed and aerated yeast—less in combination. Each inhibits the uptake of the other by respiring yeast. Sulfate reduces mortality and decreases selenium content of the livers of selenate-injected rats. Selenium transport into any cell could probably be controlled if the selenium compounds involved were known. Control of selenium content of plants by fertilizers would be difficult to achieve but offers promise. However, there is little basis for hope that the feeding of any sulfur compound can be used to control selenium poisoning in animals.

Of the many approaches to the selenium problem in the past 20 years, those offering the greatest promise have to do with rendering the selenium in the soil unavailable to plants. Hurd-Karrer (*4*) in her work on the antagonism between selenate and sulfate, found that sulfate inhibited uptake of selenate by the growing plant.

The effect of sulfate and sulfur compounds on the growth and selenate uptake of organisms in selenate-containing media have been reported for a variety of organisms including yeast (*2*), *Chlorella* (*7*), and *Aspergillus* (*9*). Postgate (*6*), working with *Desulfovibrio*, studied the competitive reversal by sulfate of selenate inhibition of sulfate reduction. All of this work appears to support Hurd-Karrer's hypothesis—that these ions compete for some reactive site.

Phosphate reduces the toxicity of selenite to yeast but there is no apparent effect on selenate toxicity (*1*). The relationship between phosphate, sulfate, and selenate was investigated in an effort to establish some factor or process common to the metabolism of selenite and selenate. Hilz and Lipmann (*3*) suggest that the first step in the metabolism of sulfate is the formation of "active sulfate." The two terminal phosphates of adenosine triphosphate would be removed as pyrophosphate and replaced by a single sulfate.

Method

Work with yeast was carried out on suspensions with a total volume of 3 ml. containing 1.5 mg. of wet baker's yeast (Fleischmann). The pH was maintained at about 3 by a 0.1M glycine–hydrogen chloride buffer which was 0.02M in potassium chloride with glucose as substrate. In most cases, two series of flasks were set up on a standard Warburg apparatus, maintained at 31° C., one series having potassium hydroxide in the center and the other, water. This gave a measure of both oxygen uptake and fermentation carbon dioxide.

In the injection work, male albino rats of the Sprague-Dawley strain were grown on standard laboratory rations and injected when they weighed between 150 and 200 grams. Sulfate injections were made subcutaneously in one flank about 10 minutes before the selenate was injected in the opposite flank. Handling of the selenium-75 injections and subsequent determination of activity were the same as reported previously (*5*). Sodium salts were used routinely. Radioactive potassium selenate was obtained from the Oak Ridge National Laboratory for use in the tracer studies.

Results

When yeast suspensions were prepared from baker's yeast that had been washed and aerated at room temperature overnight, $3 \times 10^{-4}M$ sulfate inhibited oxygen uptake to a considerable degree. This made it necessary to vary the treatment of the yeast somewhat. If yeast were suspended in 1% glucose solution and shaken for an hour, subsequent washing and overnight aeration did not render it susceptible to inhibition by sulfate. If $1 \times 10^{-3}M$ phosphate was added to suspensions prepared from this yeast, oxygen uptake was strongly inhibited by sulfate. Neither phosphate nor the glucose pretreatment affected selenate inhibition. This pretreatment was used routinely for the isotope studies.

One feature of the inhibition of oxygen uptake produced by sulfate is that it can be reduced by selenate—i.e., when $3 \times 10^{-4}M$ sulfate and $1 \times 10^{-4}M$ selenate are added to washed and aerated yeast, the oxygen uptake for the combination is higher than for the flasks with either ion alone (Figure 1). In this figure also can be seen the effect of aeration with glucose prior to washing and overnight aeration, the combination of sulfate and selenate being almost noninhibitory to the pretreated yeast. Data for the effect of sulfate and selenate on the pretreated yeast were omitted from the graph as sulfate did not inhibit, and the selenate inhibition could not be distinguished from that shown for the untreated yeast.

The effect of sulfate on selenate uptake has been studied by others (*4*, *7*, *9*); hence no particular emphasis was placed on determining competitive ratios. When activities of supernatants were compared, 15 mg. of wet yeast removed 60% of the selenate from 3 ml. of a $1 \times 10^{-5}M$ solution when aerated in the absence of added sulfate for 90 minutes. If the solution is at least $1 \times 10^{-4}M$ in sulfate, no detectable uptake of selenate occurs. The reverse process is a little hard to study, because of the toxicity of the selenate. However, radioactivity due to sulfate is decreased 90% in a $1 \times 10^{-6}M$ sulfate solution and only 20% when $2 \times 10^{-6}M$ selenate is also present. Under the same conditions, $1 \times 10^{-3}M$ phosphate had no effect on selenate uptake from $1 \times 10^{-5}M$ selenate, and $1.5 \times 10^{-4}M$ phosphate had no effect on the sulfate uptake from $7.5 \times 10^{-5}M$ sulfate.

Table I summarizes the results of two injection experiments with rats. Each experiment was carried out by injecting nine groups of five rats each, using 5, 6,

[1] Present address, Department of Chemistry, University of Portland, Portland 3, Ore.

[2] Present address, Department of Agricultural and Biological Chemistry, Pennsylvania State University, University Park, Pa.

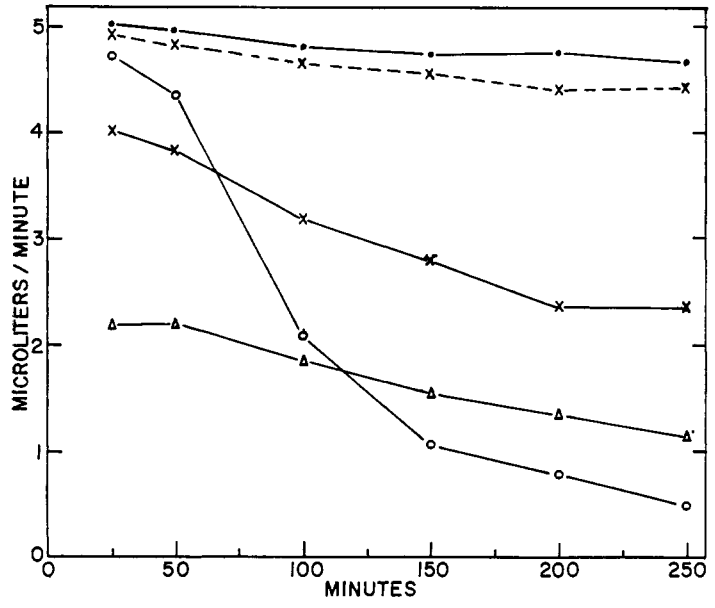

Figure 1. Effect of glucose pretreatment and added selenate and sulfate on oxygen uptake of yeast

●Control
△3 × 10⁻⁴M in added sulfate
○1 × 10⁻⁴M in added selenate
x 3 × 10⁻⁴M in added sulfate and 1 × 10⁻⁴M in added selenate
- -Aerated with glucose, then washed and aerated overnight

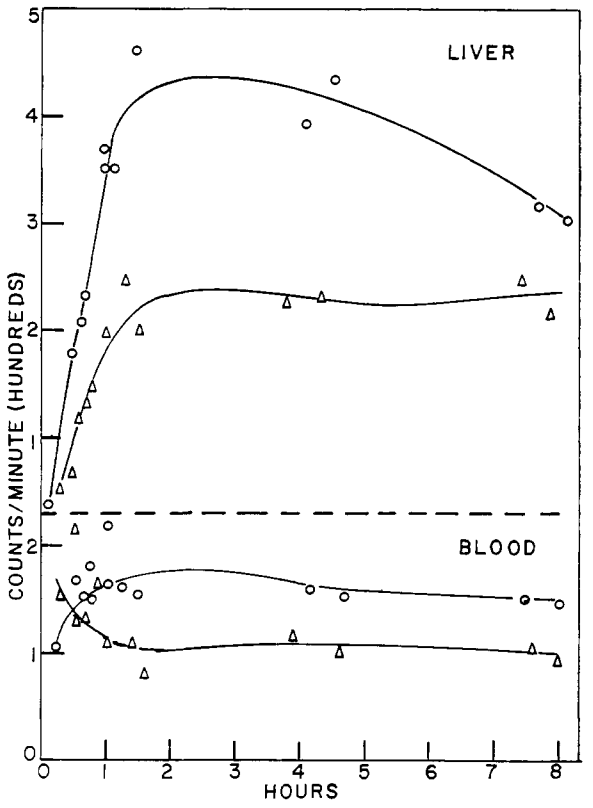

Figure 2. Effect of injected sulfate on radioactivity in liver homogenate and blood of rats injected with Se^{75} selenate at the rate of 1.5 mg./kg. of body weight

△ Rats received 2 ml. of 0.4M $MgSo_4$/kg.
○ Rats received no sulfate

and 7 mg. of selenate selenium and 0, 2, and 4 ml. of 0.4*M* magnesium sulfate per kilogram of body weight. All levels of selenate were lethal to the majority of rats, while the higher level of sulfate gave some protection against the highest level of selenate. Preliminary experiments with sodium sulfate gave similar results, but caused the animals much distress.

Two milliliters per kilogram of 0.4*M* magnesium sulfate solution altered the distribution of radioactivity when selenium-75 selenate was injected at the rate of 1.5 mg. of selenium per kg. (Figure 2). Apparently the sulfate and selenate compete for entry into the liver. The one major difference between these results and those obtained with selenite and arsenite (*5*) is the fact that, after the first hour, blood selenium-75 levels are lower in the sulfate-injected rats. Without further work to determine whether this difference is caused by blocking of selenate entry into the blood stream or by increased excretion, interpretation is difficult. Further evaluation of this work awaits the completion of studies on liver slices and determination of excretion routes.

Discussion

One of the original objectives of this work—to discover some metabolic link between selenate and selenite—has not been attained. In fact, the work offers further indications of the differences between the two. If as suggested earlier (*1*), the entry of selenite into the cell is promoted by phosphoglyceraldehyde dehydrogenase, an "active selenite" analogous to adenosine triphosphate is a possibility. This could reasonably be expected to have about the same reactivity as the selenate analog of the active sulfate of Hilz and Lipmann (*3*). If the two active intermediates were to react with the same compound, it is conceivable that metabolism of the products would follow the same route. (According to a recent paper by Wilson and Bandurski (*10*), proof of the existence of "active selenate" may reasonably be expected in the near future.)

As phosphate does not affect the uptake of sulfate, any interaction between these ions probably involves some reaction not directly involved in sulfate transport; but the antagonistic effect of sulfate and selenate on respiration seems to be due almost entirely to competition for a transport mechanism.

The interference of sulfate with selenate uptake is not an argument in favor of the theory that selenium toxicity is due to interference with sulfur metabolism. In the work reported here, respiration by washed yeast cells was not stimulated by sulfate so it is difficult to believe that the inhibition found in those flasks, containing both selenate and sulfate, was due to interference with sulfate uptake or metabolism.

No evidence was found in the literature that could be interpreted as supporting any particular mechanism of selenium toxicity. Much of the work in support of the sulfur-selenium theory of selenium toxicity can be regarded as a study of selenate metabolism. That is, if it is assumed that any sulfur-containing metabolite that is supplied in excess will be utilized as a source of energy and the sulfur oxidized to sulfate, work with methionine (*2, 7*) and other compounds (*9*) can be looked upon as a study of sulfate-selenate antagonism. Especially in considering the work of Weissman and Trelease, where several sulfur compounds blocked selenate metabolism, the simplest explanation is based on the assumption that all these compounds were effective by virtue of conversion to a common metabolite.

Shrift (*8*) was able to block the toxicity of selenomethionine by the addition of methionine. However, he reported morphological effects of selenomethionine toxicity that he did not report for selenate (*7*). Probably the selenium

Table I. Mortality in Rats Receiving Indicated Amounts of Magnesium Sulfate and Selenate Selenium per Kilogram of Body Weight

Se, Mg.	Ml. 0.4M $MgSO_4$		
	0	2	4
5	4	1	0
	5	3	0
6	5	3	1
	5	4	0
7	5	5	0
	5	5	2

analog of any essential sulfur metabolite would be toxic to some system, but there is no proof that such toxicity is related to the problem of selenium toxicity as it affects the livestock industry.

When a mechanism acquires general acceptance, such acceptance will possibly affect the design of experiments in the field. Much that has in the past been interpreted as evidence concerning the nature of selenium toxicity may simply be evidence concerning a step in the metabolism of selenate.

In the practical control of the selenium problem, ion antagonisms show considerable promise. It will be necessary to learn more about the nature of selenium in the soil solution, but from the wide variety of antagonisms discussed in this and an earlier paper (*1*), it should be possible to regulate the uptake of selenium by plants. Whether such regulation would be complete or inexpensive enough to be practical remains to be seen.

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RESISTANCE FACTOR DETERMINATION

Quantitative Estimation of the Resistance Factor, 6-Methoxybenzoxazolinone, in Corn Plant Tissue

STANLEY D. BECK, E. T. KASKE, and E. E. SMISSMAN

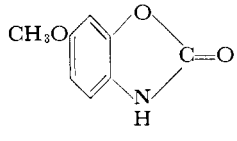
Departments of Entomology and Pharmaceutical Chemistry, University of Wisconsin, Madison 6, Wis.

A method is described for estimating the concentration of 6-methoxybenzoxazolinone in corn plant tissues. The plant tissue sample is extracted with diethyl ether, and the ether extract is purified, chromatographically, with aluminum oxide. The compound is eluted from the aluminum oxide by an ether-alcohol solvent mixture, and its concentration is estimated by its extinction at 285 mμ.

An organic compound, $C_8H_7O_3N$, was recently isolated from the tissues of the corn plant by Loomis (*6*). Independently and nearly simultaneously, the same substance was isolated from wheat and corn by Virtanen, Hietala, and Wahlroos (*11*), who tentatively identified it as 6-methoxybenzoxazolinone (I). Subsequent work by Virtanen and coworkers (*5*, *12*) and the authors' group (*8*, *9*) has established the validity of this characterization. The demethoxy analog was also isolated by Virtanen and Hietala (*10*) from rye plants. Virtanen and coworkers discovered these substances during the course of a study of antifungal chemicals present in plants of the grass family. The work of Loomis came as an outgrowth of the earlier finding of Beck (*3*), that young corn plants contain substances inhibiting the growth of European corn borer larvae, *Pyrausta nubilalis* (Hbn.). 6-Methoxybenzoxazolinone, termed resistance factor A (RFA), acts as a growth inhibitor toward a variety of bacteria, fungi, and insects in addition to the European corn borer (*1*, *7*).

The role of chemical factors in the resistance of corn plants to successful attack by the European corn borer has been studied in some detail (*1*, *2*, *4*, *7*). In addition to the ether-soluble resistance factor A, a water-soluble borer and fungus inhibitor has been detected, but not yet isolated, which has been designated as resistance factor B (RFB) (*4*, *7*). The existence of a third factor (RFC) was found after the analytical method described below was developed (*2*). Like factor A, factor C is ether-soluble, but it has not yet been isolated and characterized. Factor A is primarily responsible for the resistance of young corn plants to the leaf-feeding stage of the borer (*2*, *7*). Tissue concentrations of factor A tend to be high in young plants, but decline rapidly as the plant matures (*2*, *7*). Corn which has tasseled no longer contains factor A in amounts which are effective against either fungi or European corn borer (*1*, *2*). Resistance manifested by corn plants, which have reached the tassel growth stage, appears to be causally related to the concentrations of factors B and C in those tissues attacked by the insect (*2*).

The importance of these chemical factors in plant resistance to insects and disease makes them of considerable biological and chemical interest. The only methods available for estimating the presence and concentration of the resistance factors in plant tissues have been bioassays employing either fungi or insects (*4*). The bioassay techniques require relatively large samples of plant tissues, and are not sufficiently specific for many purposes. Such difficulties hamper detailed physiological and genetic studies. As 6-methoxybenzoxazolinone has been shown to play an important role in the defense of young corn to disease and insects, a simple quantitative method of analysis should be of value in agronomic screening of genetic lines of corn and in studies of the biochemistry of plant metabolism.



(I)

The analytical method developed is based on a chromatographic purification of resistance factor recovered from plant tissue and an estimation of its concentration from the absorbance observed at a wave length of 285 mμ. Loomis (*6*) found that purified 6-methoxybenzoxazolinone displayed two absorption peaks in the ultraviolet; one peak was at 228 mμ and the other at 285 mμ. Absorption was at a minimum at 255 mμ. The data presented in Figure 1 show that extinction at either 228 or 285 mμ (Beckman DU spectrophotometer) was linear with concentration of highly purified 6-methoxybenzoxazolinone. In