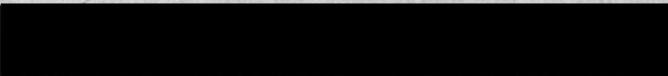


AN ABSTRACT OF THE THESIS OF

Irving Gordon Fels for the Ph.D. in Biochemistry
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Title The Role of Methionine and Sulfate in Selenate
Detoxication

Abstract Approved 
(Major Professor)

The inhibition of yeast (Saccharomyces cerevisiae) growth by selenate has been found to be reversed by the addition of methionine. No other organic compound tested displayed this activity. Within limits, the growth of yeast in the presence of selenate is directly proportional to the methionine present. Of the optical isomers of methionine only the naturally occurring L-form is effective. A methionine free casein hydrolysate was prepared and found inactive. The addition of methionine rendered it effective.

Yeast cannot utilize homocystine plus a methyl donor such as choline, betaine or creatinine in place of methionine. This suggests that the latter substance is synthesized by yeast in a different manner from that in mammalian tissue.

A scheme for the synthesis of methionine from sulfate in yeast is suggested. It is substantiated by the behavior of sulfate, methionine and p-aminobenzoic acid in selenate and sulfonamide inhibition. A relatively constant ratio has been found for a given concentration of selenate causing inhibition, and for the amount of sulfate necessary to reverse it. This suggests that the mode of action of selenate toxicity in yeast is a competitive inhibition between sulfate and selenate resulting in the blocking of methionine synthesis. This is operative when sulfate is the sole source of sulfur.

The role of methionine in reversing selenate toxicity has been investigated. Evidence is presented which makes it improbable that the detoxication occurs via an oxidation of methionine to sulfate, or a methylation by methionine.

Methionine has been found to react readily with H_2SeO_4 or SeO_2 in vitro. Crystalline compounds containing Se, N, and S have been isolated and their preparation is given.

Thiamin has been found to be the only substance of those tested which is capable of enhancing the reversal of selenate by methionine.

In E. coli, cysteine, and to some extent glutathione, can effectively counteract selenate. Methionine does not exhibit such action. Evidence is presented which indicates that the synthesis from sulfate is accomplished by different routes in E. coli and Saccharomyces cerevisiae.

Previous proposals to explain the toxicity of selenium compounds are discussed and shown not applicable for yeast. The opinion is expressed that the toxicity of selenium compounds is a multiple one in which several systems are attacked simultaneously. Two of these systems involve methionine and thiamin and their utilization. A third toxic effect is the intracellular accumulation of an insoluble, non diffusible substance which is probably elemental selenium. This is demonstrated photographically. A discussion is given which attempts to explain the specificity of methionine action.

THE ROLE OF METHIONINE AND SULFATE
IN SELENATE DETOXICATION

by

IRVING GORDON FELS

A THESIS

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APPROVED:

[Redacted Signature]

Professor of Chemistry

In Charge of Major

[Redacted Signature]

Head of Department of Chemistry

[Redacted Signature]

Chairman of School Graduate Committee

[Redacted Signature]

Dean of Graduate School

C. W. 7/1/49

CHARLES BROWN PAPER
ADVANCE BOND

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THE ROLE OF METHIONINE AND SULFATE
IN SELENATE DETOXICATION

SECTION I. Introduction

The physiological behavior of selenium compounds has evoked interest for more than a century. In the beginning, this could be attributed to a scientific curiosity which led the early workers to study qualitatively the effects of various selenium compounds upon animals. Later, however, the problem assumed practical and economic aspects when it was found that selenium was responsible for the death of farm animals suffering from "alkali disease". Although these early experiments were for the most part crude in nature, the toxicity of selenium compounds was generally recognized and believed to be similar to that of arsenic (1,2).

The earliest toxicological study of selenium salts can be traced back only with difficulty. This is in large part due to inaccurate citations present in the early literature in which investigations with tellurium are confused with those of selenium. Thus Jones (3) ascribes to Gmelin (4) the earliest recognition of the toxic effect of selenium salts on animals. A similar claim for Gmelin has recently been made by Sneider (5). Although it is true that Gmelin did study the

toxicological effects of numerous substances, selenium compounds were not included among them. The confusion probably originated from the latter's investigations with tellurium. The administration of tellurium oxide to dogs was found to be toxic. Elemental tellurium was deposited on the intestinal walls and the animals emitted a garlic like odor. A similar effect was observed by Hansen (6) who considered the volatile compound to be ethyl telluride purely on olfactory evidence. Hofmeister in 1894 (7), passed the expired air from dogs receiving injections of sodium tellurite or tellurate, through iodine in aqueous KI. The iodine was removed with KOH and the addition of Na_2S yielded an odor resembling dimethyl sulfide. Elemental tellurium was obtained from the solution by reduction. From this evidence it was concluded that the volatile compound was dimethyl telluride, H_2Te being excluded owing to its different odor. In an analogous manner, Hofmeister considered dimethyl selenide to be formed from selenium salts, although no experiments were performed to confirm this. It is interesting to note that Hofmeister suggested that the methyl groups originated from choline, creatine and other methyl donors. The concept of methylation being a general metabolic process thus finds its origin in this early study.

Maasen (8) repeated Hofmeister's work using selenium salts with dogs as well as with Penicillium brevicaulis. The conclusion was reached that dialkyl selenides were responsible for the odoriferous compounds and that the animal exerts a methylating action whereas the mold utilizes an ethylation. Although the evidence again rested upon the presumably characteristic odors of these compounds, this opinion was strengthened by Biginelli's work on the arsenical (Gosio) gas (9) which he believed to be diethyl arsine but which has recently been shown to be trimethyl arsine (10). Whereas dimethyl selenide has been isolated from mold cultures incubated with selenium salts (11), similar verification in animals has yet to be established. Schultz & Lewis (12) in recent studies of respiratory elimination of volatile selenium, were able to obtain 17 to 52 percent of injected selenite in rats in the form of volatile compounds. In a similar study using radio-active selenite, McConnell (13) was able to obtain only 3 to 10 percent of the selenate administered. In either case, no identification was attempted.

The toxicity of selenium compounds has given rise to several hypotheses in an attempt to explain its physiological action. Rabuteau (14) ascribed the death of an animal fed Na_2SeO_3 as due to mechanical blocking by

selenite crystals and elemental selenium which he found in large quantities in the blood. This observation could not be verified by subsequent workers (15,16). Because selenite has been found more toxic than selenate (3,17-25), it is generally believed that selenate must first be reduced to selenite before any toxicity is manifested (16). Elemental selenium itself is innocuous (15,26). The early workers (3,15,16,22,27) were unable to demonstrate any individual toxicological action of selenium compounds on the cells of the animal organism, but concluded that these acted deleteriously on the animal body as a whole. With the knowledge that H_2SeO_3 and H_2SeO_4 react readily with thiol compounds, Bersin (28) in 1935 proposed that selenite and selenate react with glutathione to give an intermediate of the type $Se(SR)_2$. It had been shown previously that both selenite and selenate could catalyze the oxidation of glutathione (29). The cysteine analogs of this type compound have recently been prepared (30,31).

It was a logical sequence from this to assume that selenium compounds inhibit certain enzymatic reactions dependent upon reversible sulfhydryl-disulfide changes and those systems which require the presence of free sulfhydryl groups. Bersin & Logemann (32) believed that the inactivation of papain by selenate was due to the

oxidation of the sulfhydryl groups. Collett (33) found that selenite inhibited frog succinoxidase. The same author and co-workers reported selenite to be toxic to citrase and lactase in frog muscle (21). The same reagent had no effect upon L-malase and glycerophosphorase; nor did selenate exert any influence on most of the dehydrogenases. The inhibition of pig (34) and chick (18,35) succinoxidase have been reported. As a result of the observation that selenite does not cause as complete inhibition of O_2 uptake in yeast as does cyanide, Potter & Elvehjem (19) suggested that selenite does not inactivate the cytochrome system but rather attacks dehydrogenating mechanisms. From the observation that the respiration of glucose was 80% inhibited by selenite and 4-9% when lactate and pyruvate were used, these authors believed that selenite attacked the glycolytic system and that its reaction with glutathione offered the best explanation for its toxicity. An observation which thus far has escaped attention is the inhibition of phosphatase by selenite and selenate (36).

In addition to the methylation mechanisms proposed by the early workers by which the animal organism was thought to nullify the toxic effects of selenium compounds, several others have been enunciated although often these have not been supported by sufficient

evidence. The observation that glucose reacted with selenite in vitro with the production of elemental selenium, led Jones in 1909 (3) to the opinion that glucose was the cell's defense against the injurious effects of selenium salts. In support of this concept, one rat injected with Na_2SeO_3 was found to suffer a reduction in liver glycogen, accompanied by a hypoglycemia. So convinced was this author in the correctness of his view based upon this evidence, that he actually used selenite injections on a diabetic patient in an attempt to prove that diabetes is not due to any lack or difficulty in breaking up the glucose molecule. This he was able to do with the same degree of thoroughness. Another unique experiment was performed by Cathcart & Orr (37) who used one dog both as the control and test animal. The conclusion was reached that the effect of selenite was more marked on a low carbohydrate diet than on a high one. Similar results obtained by Jones and Cathcart & Orr have been claimed by Levine & Flaherty (38) in a report containing little data. In contrast to these findings, Pellegrino & Gaizzone (39) in a carefully controlled study, record that the intramuscular injection of selenic acid into rabbits resulted in all cases in an increase in blood sugar. These discrepancies have recently been clarified by Wright (40). It was found

that an increase in blood sugar accompanied by a decrease in liver glycogen was obtained upon intravenous injection of selenite into well fed rats or rabbits. No such effect was observed in fasting animals which would occasionally exhibit a hypoglycemia when in a semi-moribund condition.

Other views on the formation of physiological inert selenium have been forwarded. The reduction of selenite to selenium by bacteria was stated by Klett (41) to be proportional to the growth and a product of cellular activity. Jones (3) incorrectly attributed to Hofmeister (7) the belief that the reduction process is a direct chemical reaction and not enzymatic. Since the latter author studied the toxicological effect of tellurium salts and not selenium, these statements originate with Jones and are without experimental support. In another paper which contains no experimental data, Levine (25) stated that both plant and animal tissues fail to reduce selenite after heating, the exception being fresh liver which can produce elemental selenium regardless of the heating treatment. It was concluded that the reduction was caused by an endoenzyme. More recently it has been quantitatively demonstrated (17) that selenate is converted into selenite and volatile selenium compounds by beef liver whereas autoclaved liver is unable to effect

tubes with a sterile towel instead of the usual cotton plugs. For an inoculum, four to five loopfuls of yeast from a freshly grown slant were homogeneously suspended in 15 ml. of sterile saline. This was centrifuged and resuspended in another 15 ml. of saline. The yeast concentration as determined turbidimetrically was approximately 0.8 mg. per ml. on a wet weight basis. One drop of this suspension was used as the inoculum, although it was found that the size of the inoculum was not critical; five drops yielded the same results as one. The tubes were incubated at 30°-31° for two to three days and the turbidity was measured in a photoelectric turbidimeter with a 5400 A filter. Growth was expressed in terms of optical density which is equal to $\log 100$ minus \log percent transmission ($2 - \log G$).

L-cysteine^c, glutathione^c, DL-methionine^d, thymine, cytosine, guanosine, guanylic acid, adenosine, adenylic acid, 2-methyl-6-amino pyrimidine, 2-methyl-5-ethoxymethyl-6-amino pyrimidine, hydrolyzed desoxyribonucleic acid^e, as prepared by Levene and Bass (54), and 200 micrograms of each of the following: 2-amino-4-hydroxy-6-methyl pyridine, 2-amino-4-hydroxy pyridine-6-carboxylic acid, and pteric acid.

The relationship of DL-methionine to selenate inhibition is depicted in Table II and shown graphically in Fig. I. Straight lines are obtained when the plot is made on a logarithmic scale. Concentrations of methionine above 3 mg. per tube did not enhance the reversal.

In view of the fact that animals can utilize homocystine plus a methyl donor such as choline or betaine (59) in lieu of methionine, an attempt was made to determine whether yeast can use these substances to reverse the effect of selenate. No activity was noted with 2 mg. of DL-homocystine, choline, betaine, or creatinine, either individually or in combination.

c L-cysteine and glutathione reacted with H_2SeO_4 prior to inoculation but no reversal was evidenced.

d Kindly furnished by U.S. Industrial Chemicals Inc.

e Obtained from the Krishell Laboratories, Portland, Oregon.

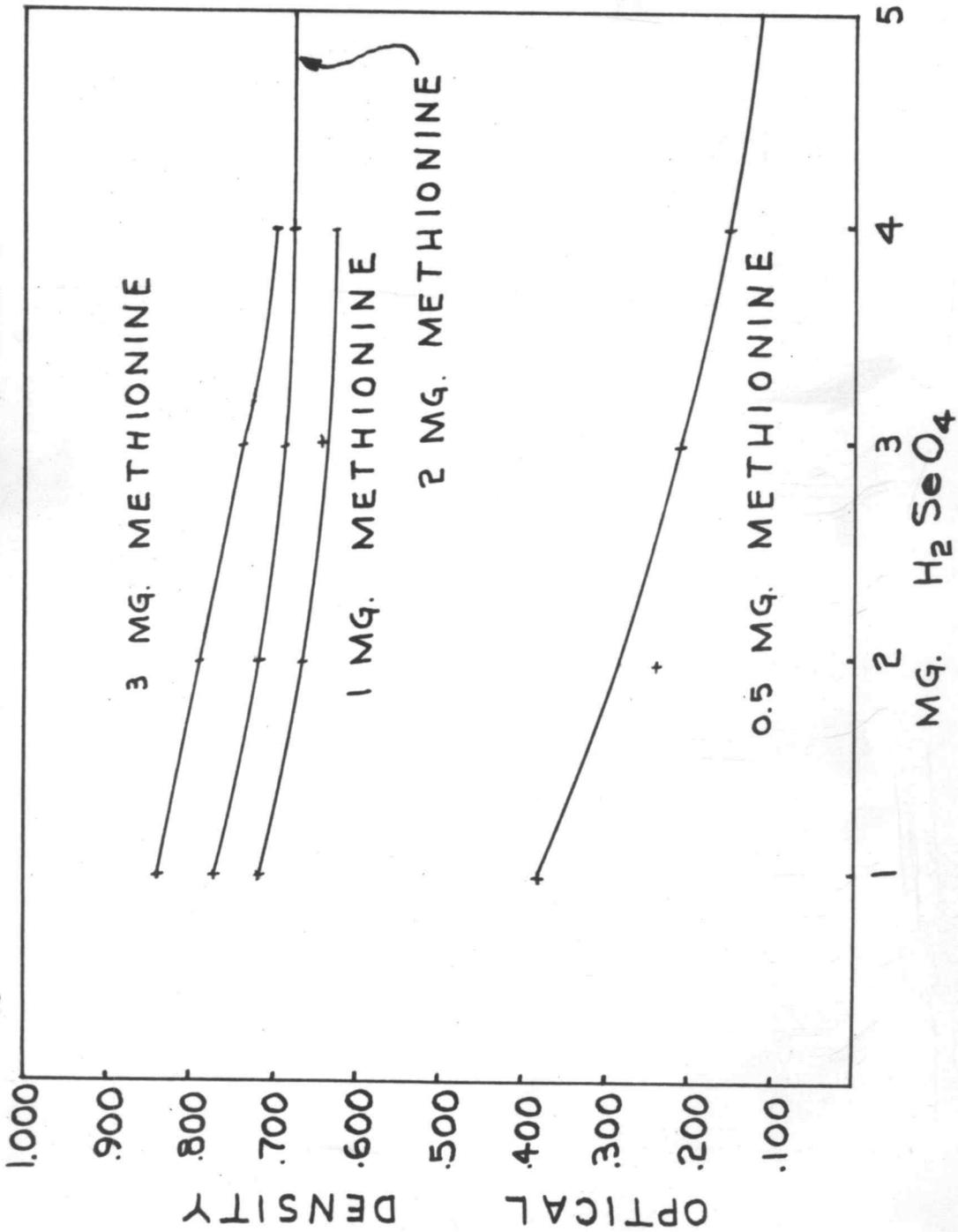
Table II.

Relationship of DL-Methionine to Selenate
Inhibition in Saccharomyces cerevisiae

<u>H₂SeO₄</u> mg.	<u>Methionine</u> mg.	<u>Optical Density</u>
0	0.0	0.980
1	0.0	0.000
1	0.5	0.381
1	1.0	0.718
1	2.0	0.770
1	3.0	0.840
2	0.0	0.000
2	0.5	0.232
2	1.0	0.660
2	2.0	0.732
2	3.0	0.785
3	0.0	0.000
3	0.5	0.212
3	1.0	0.640
3	2.0	0.650
3	4.0	0.708
4	0.0	0.000
4	0.5	0.164
4	1.0	0.631
4	2.0	0.680
4	3.0	0.702
5	0.0	0.000
5	0.5	0.125
5	2.0	0.675

Incubation time: 52 hours

Figure I. Reversal of Selenate Inhibition by Methionine



Action of Methionine Free Casein Hydrolysate

The specificity of methionine reversal was also tested by noting the effect of a methionine free casein hydrolysate upon selenate activity. The hydrolysate was prepared as follows, with a modification of Baernstein's method for determining methionine in protein hydrolysates (60):

500 mg. of Casamino acids (Difco) were refluxed with 25 ml. of freshly distilled HI (sp. gr. 1.7) for 8 hours in a CO₂ atmosphere. The excess HI was removed by vacuum distillation, a few drops of dilute HCl being used to facilitate removal. A silver chloride suspension (prepared by precipitating Ag₂O from AgNO₃ and NaOH, centrifuging, decanting the supernatant and adding strong NCl to form AgCl) was added in excess to the hydrolysate. This was added to a large centrifuge tube, shaken for 10 minutes, centrifuged and filtered. The precipitate was washed twice and the washings were added to the filtrate. The latter was evaporated to dryness and reconstituted to 50 ml. The final concentration was thus 10 mg. per ml. The filtrate gave a negative McCarthy-Sullivan test for methionine

The effects of this hydrolysate, with and without supplementation with DL-methionine are summarized in Table III. No reversal of selenate inhibition was

obtained with this preparation, but when DL-methionine was also added the growth of yeast seemed fully restored. Although the levels employed do not permit a precise calculation of the reversing power of ordinary casein hydrolysate in terms of methionine content, it is evident that the casein effect is due principally to methionine.

Specificity of L-Methionine in Reversal of Selenate

The postulate has recently been made (17) that there is present in mammalian tissue a heat labile factor or factors, presumably an enzyme, which is responsible for the decomposition of selenate and selenite. This is based on the observation that fresh tissue can decompose selenium salts whereas autoclaved tissue is incapable of such action. The present author has obtained similar results with yeast. This would appear to lend to the cell an active role in the detoxication mechanism, in which methionine might cooperate. In order to gain evidence on this point, DL-methionine was resolved into its optical isomers and each was tested for its ability to reverse the selenate effect.

The resolution of DL-methionine was performed according to the method of Windus and Marvel (61) in which methionine is formylated with anhydrous formic acid and the formyl derivatives of the two optical isomers are separated as the brucine salt. The optical rotations of

the D and L-methionine obtained are given below with values already present in the literature:

Rotation: 0.2000 gms. of D-methionine in 25 ml.
 H₂O; 2 decimeter tube at 25° C.; = +0.11°
 = +6.88°
 Windus and Marvel (61) = +8.12°
 Jackson and Block (62) = +7.1° (average)

0.2000 gms. of L-methionine in 25 ml.
 H₂O - 0.08° = -5.0°
 Windus and Marvel = -7.5° and -6.9°
 Jackson and Block = -7.3° (average)

Both the D and L-isomers gave a positive McCarthy-Sullivan test, but only the L-form could be utilized by Lactobacillus arabinosus (63) with Henderson and Snell's medium for methionine determination (64).

The results listed in Table IV show clearly that only the naturally occurring isomer of methionine is capable of reversing the inhibition by selenate, although the racemic mixture is somewhat more than 50 percent as active as equal amounts of the L-form. This might be due to incomplete resolution; but if not, it suggests that the natural isomer promotes the utilization of the unnatural one, an observation which has been made for glutamic acid in Lactobacillus arabinosus (65).

The specificity of methionine in the detoxication of selenate by yeast is first apparent from its direct relationship as depicted in Fig. I. Within limits, the growth of yeast in selenate is directly proportional to the amount of methionine present. That methionine is the only substance capable of such action is seen both from the numerous substances tested and the experiments with methionine free casein hydrolysate. It is interesting to note that unlike animal tissues, yeast is incapable of utilizing homocystine plus a methyl donor such as choline, betaine, or creatinine. This would indicate that methionine is synthesized by yeast in a different manner from that accomplished by animals. The fact that yeast is able to synthesize its sulfur containing amino acids from the sulfate provided lends support to this concept.

Table III.

Effect of Methionine Free Casein Hydrolysate
Upon Selenate Inhibition in Yeast

<u>H₂SeO₄</u> mg.	<u>DL-Methionine</u> mg.	<u>Methionine Free</u> <u>Hydrolysate</u> mg.	<u>Casein</u> <u>Hydrolysate</u> mg.	<u>O.D.*</u>
0	0	0	0	1.32
1	0	0	0	0.00
1	2	0	0	1.03
0	0	10	0	1.27
1	0	10	0	0.00
1	2	10	0	1.16
0	0	0	28.5	1.48
1	0	0	28.5	0.41
1	2	0	28.5	1.34

Incubation time: 71 hours

* Optical Density

The results obtained with the optical isomers of methionine indicate that the cell plays an active role in the detoxication mechanism. This is in line with the recent work of Rosenfeld and Beath (17). Another interpretation of the present findings may be expressed in terms of the prevention of methionine utilization, which like cellular detoxication would involve a specificity of a type associated with enzymatic activity. Further discussion of this point will be reserved for a later section (Section VII).

The suggestion of Potter and Elvehjem (19) that selenium salts act primarily in restricting the utilization of glutathione, finds no support in the present experiments with yeast. Although DuBois et al. (66) have been able to relieve the toxic effect of selenate in rats with glutathione, this has not been observed with yeast. It would seem that since the function of glutathione is at present unknown, an explanation of selenate toxicity in terms of glutathione is of limited value. From a practical point of view, it is of interest to note that methionine affords protection against selenite in rats (67). No such effect has been observed with cystine (67,68,5).

Table IV.

Specificity of L-Methionine in Selenate Inhibition

<u>H₂SeO₄</u> mg.	<u>DL-</u> <u>Methionine</u> mg.	<u>O.D.*</u>	<u>L-</u> <u>Methionine</u> mg.	<u>O.D.</u>	<u>D-</u> <u>Methionine</u> mg.	<u>O.D.</u>
0	0	1.120				
1	0	0.000				
1	0.25	0.494	0.25	0.558	0.25	0.000
1	0.50	0.824	0.50	0.881	0.50	0.000
1	1.00	0.839	1.00	0.910	1.00	0.000
1	2.00	0.980	2.00	0.981	2.00	0.000
2	0.25	0.187	0.25	0.252	0.25	0.000
2	0.50	0.770	0.50	0.980	0.50	0.000
2	1.00	0.805	1.00	0.850	1.00	0.000
2	2.00	0.860	2.00	0.920	2.00	0.000
3	0.25	0.000	0.25	0.045	0.25	0.000
3	0.50	0.070	0.50	0.144	0.50	0.000
3	1.00	0.655	1.00	0.720	1.00	0.000
3	2.00	0.710	2.00	0.760	2.00	0.000

Incubation time: 67 hours

* Optical Density

SECTION IV. The Role of Sulfate in Selenate Toxicity*

In the previous section it was seen that the selenate toxicity in yeast could be partially reversed by methionine. This seemed to bear a superficial resemblance to the reversal of sulfonamides by methionine (69). The fact that methionine must be synthesized from sulfate in a medium utilizing this anion as the sole sulfur source, in addition to the present belief that p-amino-benzoic acid (PABA) is concerned in methionine synthesis, (69,70) suggested a relationship between the action of selenate toxicity, sulfonamide inhibition and methionine synthesis.

Fig. II. represents a scheme which was devised to relate the effect of selenate and sulfonamides, as well as the possible position of selenate in prevention of methionine synthesis. The sequence $SO_4 \longrightarrow X$ represents a series of precursors of methionine derived from sulfate, and the sequence $\longrightarrow Y$ represents precursors arising from a carbon source. The branching scheme was employed because the synthesis of methionine requires contributions from both sources. Since animals do not

* The content of this section was presented before the Division of Biological Chemistry, American Chemical Society, 114th meeting, Portland, Oregon, September 1948, and published in Archives of Biochemistry. (in press)

utilize sulfate appreciably for synthetic purposes (71), PABA was considered to act on the carbon precursors. Selenate, on the other hand, was placed in direct relation to sulfate because of the chemical similarity of the latter two substances.

According to the above hypothesis, it should be possible to inhibit the synthesis of methionine with sulfonamides and counteract the inhibition with PABA, but not with sulfate. Similarly it should be possible to inhibit the organism with selenate and reverse the inhibition with sulfate but not with PABA. On the other hand, methionine should be capable of reversing the inhibition caused by both inhibitors. The experiments described below appear to substantiate this hypothesis.

Experimental

The methods, organism and medium were similar to that described in the previous section with the difference that the sulfate was eliminated from the medium as completely as possible. Figure III represents the growth response curve, obtained by adding increments of sulfate to the cultures. The concentration of sulfate necessary to permit half maximum growth for a given concentration of selenate is shown in Table V. The molar inhibition ratio,

Fig. II.

Suggested Points of Action of Selenate and Sulfonamides in the Biosynthesis of Methionine From Sulfate by Yeast

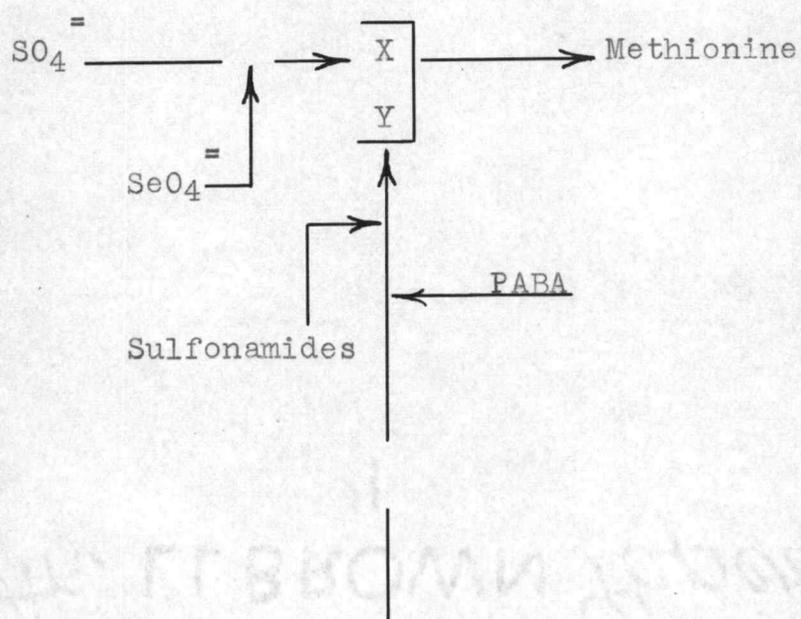
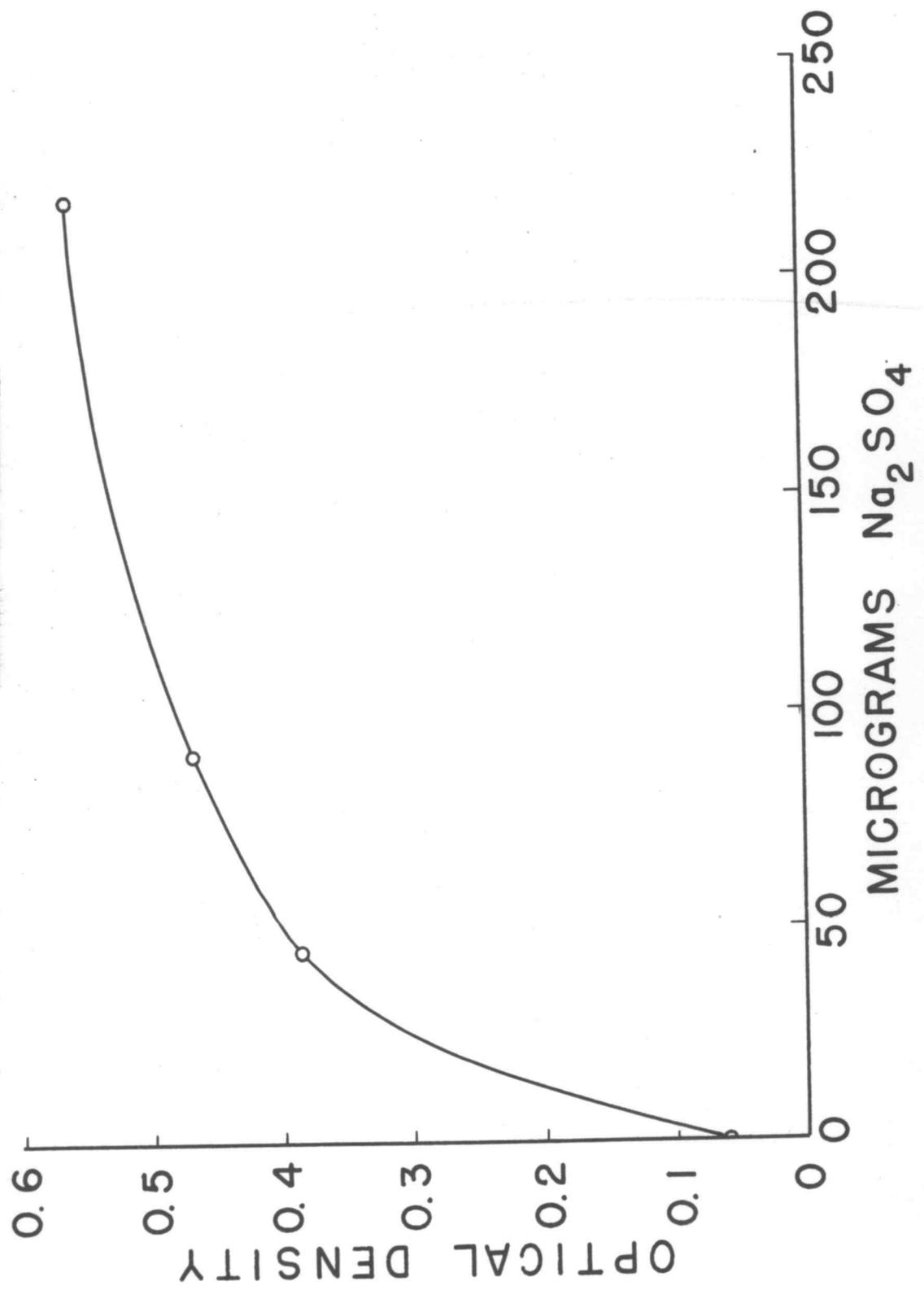


Figure III. Growth Response of Saccharomyces cerevisiae to Added Sulfate



I/S, as determined by the method of Shive and Macow (72) is also included. The sulfate used was in the form of $\text{Na}_2\text{SO}_4 \cdot 10\text{H}_2\text{O}$, although the calculations were made on the basis of the anhydrous salt.

The influence of sulfate, methionine and PABA upon sulfonamide inhibition is given in Table VI. The medium used here contained 0.5 gm. $\text{Na}_2\text{SO}_4 \cdot 10\text{H}_2\text{O}$ per liter. It was identical in other respects to that mentioned above. Concentrations of PABA greater than those listed in Table VI were found to be inhibitory. 2-chloro-4-aminobenzoic acid#, which is considered to be a specific inhibitor of methionine synthesis in Escherichia coli (70,73,74), was also used in these experiments, but no inhibition was noted in concentrations up to 4 mg. per tube.

The data presented indicate that a relatively constant ratio exists between the inhibitor selenate (I) and the substrate (S) necessary to counteract it. This ratio (about unity for half maximum growth) is direct evidence (72) for the existence of a competition between selenate and sulfate for an enzyme surface, and it favors the placement of these two substances in direct opposition as in Fig. II. It may be inferred from this, and from the

The author is grateful to Dr. F. B. Strandskov for a sample of 2-chloro-4-aminobenzoic acid.

ability of methionine to reverse selenate inhibition that the mode of action of selenate toxicity in yeast is the blocking of methionine synthesis from sulfate. This confirms and extends observations in plants in which sulfate was found to counteract selenate toxicity (43,44).

It should be emphasized that this mode of action is operative only when sulfate is the sole source of sulfur. From the experiments with methionine it is believed that processes other than the synthesis of this amino acid are concerned, since complete reversal of selenate toxicity is never realized with methionine.

In other experiments, PABA, in quantities up to 2 mg. per tube was unable to influence the toxicity of selenate. This is in keeping with the postulated scheme. In addition the results obtained with sulfate, methionine and PABA in sulfonamide inhibition further substantiate the correctness of this scheme. Similar results with methionine and PABA have been obtained with E. coli (69). The complete inhibition of yeast by 2 mg. of sulfonamide and its reversal by PABA has been previously reported (75). The inability of 2-chloro-4-aminobenzoic acid to produce inhibition in yeast suggests that the synthesis of methionine in yeast and E. coli is accomplished by different routes, since the growth of the latter organism is readily curtailed by this

reagent. Further evidence along this line will be presented in Section VI.

Table V.

Molar Ratios of Selenate-Sulfate Concentrations
at Half Maximum Growth of Sacc. cerevisiae (FB Strain)

<u>H₂SeO₄</u> mg.	<u>Na₂SO₄*</u> mg.	<u>I/S (Molar)</u>
0.50	0.26	1.9
0.50	0.37	1.3
1.00	1.14	0.93
1.00	1.17	0.88
1.00	1.54	0.67
1.50	0.93	1.60
1.50	1.70	0.90
1.50	1.66	0.92
2.00	1.37	1.40
2.00	1.37	1.40
4.00	2.28	1.70
4.00	3.27	1.20
		Av. 1.1

Incubation time: 24 hours

* Calculated as anhydrous salt

Table VI.

The Influence of Sulfate, Methionine and PABA
Upon Sulfonamide Inhibition in Sacc. cerevisiae

<u>Sulfonamide</u> mg.	<u>Na₂SO₄·10H₂O</u> mg.	<u>Methionine</u> mg.	<u>PABA</u> mg.	<u>O.D.*</u>
0	0	0	0	0.67
1	0	0	0	0.20
1	1	0	0	0.18
1	2.5	0	0	0.19
1	10.0	0	0	0.17
1	0	0.6	0	0.57
1	0	1.0	0	0.60
1	0	2.0	0	0.61
1	0	3.0	0	0.60
1	0	0	0.1	0.66
1	0	0	0.5	0.68
2	0	0	0	0.09
2	1	0	0	0.08
2	2.5	0	0	0.08
2	10.0	0	0	0.08
2	0	0.6	0	0.37
2	0	1.0	0	0.38
2	0	2.0	0	0.39
2	0	3.0	0	0.51
2	0	0	0.1	0.65
2	0	0	0.5	0.66
3	0	0	0	0.07
3	1	0	0	0.06
3	2.5	0	0	0.06
3	10.0	0	0	0.07
3	0	0.6	0	0.18
3	0	1.0	0	0.18
3	0	2.0	0	0.22
3	0	3.0	0	0.24
3	0	0	0.1	0.66
3	0	0	0.5	0.67

Incubation time: 24 hours

* Optical Density

SECTION V. The Role of Methionine in Selenate Toxicity

Because of the specific nature of the reversal of selenate toxicity by methionine, an attempt was made to obtain information of this action. From a logical point of view it was difficult to decide where the best point of attack of the problem could be made, since several plausible explanations suggested themselves. Furthermore, each of these explanations has a precedent in other biochemical phenomena associated with methionine. Each of these will be treated separately.

A. The Oxidation of Methionine to Sulfate

It appeared from the onset that if yeast could oxidize methionine to sulfate an explanation of the reversal process would be at hand. That such an oxidation takes place in nature is well recorded in the literature. Medes (76) found that humans oxidize methionine to sulfate approximately 100%. The oxidation to sulfate has likewise been found to take place in rabbits (77), dogs (78), plants (79) and Aspergillus niger (80). The behavior of sulfate in selenate inhibition has been discussed in the previous section.

Experimental

The first experiment was designed to investigate the oxidative capacity of "resting" yeast cells with respect to methionine. One gram of DL-methionine in a total volume of 250 ml. of dilute phosphate buffer, pH 5.2, was inoculated with a heavy suspension of washed yeast cells (about 1 mg. on a wet weight basis). This was prepared in triplicate, as were controls which contained no methionine. The flasks were then incubated at 30°C. for 24 hours with occasional shaking.

The determination of sulfate was performed according to the method of Wood and Barren (81). This entails precipitation of protein by 4% tannic acid in 1% HCl, filtration, and precipitation of the sulfate from the clear, hot filtrate by means of BaCl₂. After an overnight digestion period, the dried precipitate is weighed. Using this procedure, no sulfate could be detected either in the test or control flasks.

Oxygen Consumption in the Presence of Methionine

The possibility that only small amounts of sulfate were being formed was investigated by studying the O₂ uptake in Warburg respirometers. Washed cells (about 9 mg. on a wet weight basis) were suspended in phosphate

buffer at pH 5.2 in the presence and absence of methionine. The inner wells contained Whatman #40 filter paper impregnated with 20% KOH to absorb CO₂. The total volume of the contents in the Warburg flasks was 3.3 ml. These were then shaken for 3 hours at 30° C. at 90 cycles per minute with a 2.5 cm. amplitude. The thermobarometrically corrected oxygen consumption for this period is given in Table VII.

Experiments with Actively Growing Yeast Cells

In order not to overlook the possibility that the oxidation of methionine to sulfate might require a coupled reaction to satisfy the energy requirements of the reaction, experiments were conducted with actively growing yeast cells in a sulfate free medium containing methionine as the sole source of sulfur. The medium used is given in Table VIII. After solution, the medium was divided into six lots of 250 ml. each, sterilized by steaming for 20 minutes and inoculated with washed cells (about 1 mg. wet weight). To three of the flasks which served as controls, toluene was added. After 48 hours of incubation at 30°C., the flasks were steamed for 20 minutes and the cells separated by centrifugation. The Wood and Barren procedure for sulfate was then carried out on the clear supernatant. No sulfate was detected in any of the flasks.

Table VII.

The Oxygen Consumption of Yeast Cells in the Presence and Absence of Methionine

<u>Flask Contents</u>	<u>O₂ Uptake in cmm.</u>
1) Buffer and cells	19.6
2) Buffer, cells and 300 <i>μg</i> methionine	18.9
3) Buffer, cells and 600 <i>μg</i> methionine	20.7
4) Buffer, cells and 900 <i>μg</i> methionine	20.9
5) Buffer, cells and 600 <i>μg</i> methionine in side arm	18.9

Table VIII.

Sulfate Free Medium in Oxidation Experiments

DL-Methionine	6 gm.
Sucrose	30 gm.
NH ₄ Cl	4.5 gm.
KH ₂ PO ₄	2 gm.
CaCl ₂ ·2H ₂ O	0.4 gm.
MgCl ₂	0.4 gm.
Inositol	4 mg.
Thiamin Hydrochloride	30 μg.
Calcium Pantothenate	400 μg.
Pyridoxine	30 μg.
Biotin	0.4 μg.
Tap H ₂ O to 1500 ml.	
pH 5.2	

Experiments with Actively Growing Cells in the Presence of Selenate

These experiments were prompted by the possibility that if methionine were oxidized to sulfate, it might be accomplished only as a detoxication mechanism in the presence of selenate. The experimental conditions were similar to those described above with the exception that the test flasks contained 30 mg. H_2SeO_4 , pH 5.2, per flask, in addition to methionine. Additional control flasks containing 11 mg. per flask were also used. After 132 hours of incubation, the cells were centrifuged, washed with dilute HCl and the supernatant and washings carried through the procedure for sulfate. No sulfate was detected in any of the flasks despite the fact that sulfate had been added to the controls. A separate uninoculated control containing 11 mg. in 250 ml. of medium without selenate gave a white precipitate with BaCl_2 .

The Effect of Successive Additions of Methionine

It might be supposed from a knowledge of the competitive nature of sulfate and selenate that if methionine is oxidized to sulfate, the concentration of the latter that is formed will depend upon the concentration of selenate present. If the amount of sulfate formed is in excess of selenate, one would expect that the inhibition will be completely reversed and any excess of sulfate over

the molar ratio (selenate) / (sulfate) could be returned to synthetic channels. When by this means the sulfate concentration becomes reduced to a level where the competition is once more a factor, inhibition will once more be evidenced. Thus according to this concept, the controlling factor for growth would be the concentration of selenate present. If this hypothesis is correct, then the addition of more methionine to cells in either completely reversing the selenate or increasing the extent of the reversal.

Using the basal medium (Table I) in which sulfate was omitted, increments of sterile methionine were added to poisoned cells, and compared to appropriate controls. To keep the volumes constant, sterile distilled water was added to the other tubes. The results after 68 hours are given in Table IX. From the data presented it is apparent that successive increments of methionine do not augment the reversal process.

Despite the negative results obtained, the observation that added sulfate cannot be recovered, makes insecure the conclusion that methionine is not oxidized to sulfate by yeast under the specified conditions. The disappearance of the added sulfate in a methionine containing medium may be explained on a basis of a preferential utilization of sulfate over methionine determined possibly by differences

Table IX.

The Effect of Successive Increments of Methionine
Upon the Reversal of Selenate by Yeast

<u>H₂SeO₄</u> mg.	<u>Methionine Added</u>			<u>O.D.*</u>
	<u>Initially</u> mg.	<u>After 24 hrs.</u> mg.	<u>After 48 hrs.</u> mg.	
0	2	0	0	1.32
0	4	0	0	1.32
0	6	0	0	1.10
1	2	0	0	0.83
1	4	0	0	1.09
1	6	0	0	1.00
1	2	2	0	0.88
1	2	2	2	0.99

Incubation time: 68 hours

* Optical density

of diffusion rates into the cell. However, in attempting to detect formed sulfate, the assumption is made that such sulfate may be found extracellularly. If this does not occur, it is clear that no sulfate will be found as BaSO_4 . That the method is sufficiently sensitive to detect extracellular sulfate if it were formed, can be established from a calculation of the amount of sulfate required to counteract the selenate present. From the molar inhibition ratio of selenate and sulfate, about 28 mg. Na_2SO_4 (in 250 ml.) are required to counteract 30 mg. H_2SeO_4 for half maximum growth. This concentration is clearly within the range of detectability as BaSO_4 (solubility-0.00016 gm./100 ml. H_2O at $20^\circ \text{C}.$)

Evidence that sulfate is not formed from methionine in yeast is obtained from the oxygen uptake of cells in the presence and absence of methionine. No significant difference is observed between the control and test samples. The theoretical oxygen requirement for the oxidation of methionine to sulfate obviously depends upon the mechanism of the reaction, and since this is not known, a theoretical calculation is not possible. However, the minimum quantity of oxygen required may be calculated from the following equation:



The oxygen requirement for this reaction is the minimum amount possible in comparison to any other mechanism depicting the oxidation of methionine to sulfate. If now the theoretical O_2 requirement is calculated for the case showing the greatest deviation from the controls, i.e., 900 μ g methionine, it will be found that 0.35 mg. O_2 at S.T.P. are required to oxidize the 900 μ g methionine according to equation 1). The actual amount observed, assuming no experimental error, is 0.0016 mg. O_2 at S.T.P. If any other mechanism is assumed, considerably more oxygen will be required for the oxidation of carbon residues to CO_2 . From these calculations, it seems unlikely that any oxidation has taken place. It might be argued that the oxidation to sulfate is a relatively slow process which could not be successfully recorded in the three hour period of investigation. The negative results obtained from the experiments in which methionine was added on successive days indicate that this argument is not a valid one. Furthermore, evidence is available which makes the oxidation of methionine to sulfate by yeast highly improbable. Experiments will be described below in which the detoxication of selenate by methionine takes place under anaerobic conditions. Since the oxidation of methionine to sulfate is clearly an aerobic process, this explanation cannot be used to explain detoxication anaerobically.

B. The Methylation of Selenate by Methionine

Very pertinent to the present study is the finding of Challenger and North (11) that the mold, Penicillium brevicaulle, when incubated with bread crumbs containing selenite or selenate produced dimethyl selenide which was isolated and identified as the insoluble mercuric chloride complex. It appeared plausible that perhaps yeast detoxified selenate in a similar manner by means of methionine.

Experimental

A somewhat similar procedure was used to that devised by Challenger and North for the isolation of dimethyl selenide. Two twelve liter round bottom flasks containing six liters each of basal medium (Table I) in which sulfate was replaced by methionine (2 gms. per flask) were connected in parallel to two gas washing bottles containing 200 ml. of Biginelli's solution* (82). To each flask was added 1 gm. H_2SeO_4 after which the pH was adjusted to 5.2 and the flasks were sterilized by steaming for 45 minutes at $100^\circ C$. Upon cooling, 500 ml. of a washed cell suspension obtained from a 24 hour culture grown on the basal medium was added to each flask. All stoppers and connections were sealed with paraffin. Air,

* The composition of Biginelli's solution: 10 parts $HgCl_2$, 20 parts concentrated HCl , and 80 parts H_2O .

sterilized by bubbling through concentrated H_2SO_4 and then distilled water, was passed into the flasks by means of coarse bubblers. This was continued for ten days at room temperature. At the end of this time no precipitate was observed in the washing bottles. Growth in the flasks was accompanied by a bright orange-red coloration, which has been observed frequently in other experiments. Upon breaking the seals a foul odor became evident. The same experiment was repeated under anaerobic conditions in which nitrogen was used instead of air. Again no precipitate was formed in the Biginelli's solution. No observable differences could be detected in the rate of growth and detoxication in the two experiments. In both cases, the growth was heavy and brightly colored.

Experiments with Nitrogen Heterocycles

Nicotinic acid and nicotinamide, as well as other pyridine and quinoline derivatives are known to be methylated in the animal body (83). It appeared that evidence of methylation by means of methionine could be gained by use of these compounds in yeast. The medium used was essentially the same as the original basal medium (Table I) with the exception that one half the sulfate concentration was used. In addition, the vitamin supplements were increased in concentration to offset any

Table X.

The Vitamin Supplements of the Basal Medium

Inositol	15 mg.
Thiamin Hydrochloride	200 μ g.
Riboflavin	200 μ g.
Calcium Pantothenate	200 μ g.
Nicotinic Acid	200 μ g.
Pyridoxine	200 μ g.
p-aminobenzoic Acid	200 μ g.
Biotin	1 μ g.
Folic Acid	2 μ g.

stimulating effects of added nicotinic acid or nicotinamide. These concentrations have been used in the assay of β -alanine with the same organism (84) and are given in Table X for 1 liter of medium.

Nicotinic acid, nicotinamide, pyridine and quinoline in quantities up to 8 mg. per tube were added to the medium in the presence and absence of methionine (2 mg. per tube). Owing to their volatility, pyridine and quinoline were sterilized by filtration, and added separately after the medium had been sterilized. No inhibition was observed in any of the tubes except the higher concentrations of quinoline over periods up to 48 hours. The results of quinoline inhibition after 48 hours are given in Table XI.

The addition of more methionine (up to three milligrams per tube) also had no effect upon the inhibition by quinoline. However, it was found that growth equal to that of the controls could be obtained by longer incubation under sealed conditions. Despite the fact that in these latter experiments, the sulfate concentration was further reduced (1 mg. $\text{Na}_2\text{SO}_4 \cdot 10\text{H}_2\text{O}$ as compared to the original basal concentration of 2.5 mg. per tube), the addition of methionine was without effect and was in fact unnecessary.

From the experiments just described it does not appear that methylation by methionine is a detoxication mechanism in yeast. This is supported by the inability to detect the

Table XI.

The Effect of Methionine Upon Inhibition of
Yeast Growth by Quinoline

<u>Quinoline</u> mg.	<u>Methionine</u> mg.	<u>Optical Density</u>
0	0	0.84
4	0	0.80
6	0	0.50
8	0	0.08
4	2	0.78
6	2	0.55
8	2	0.02

glutathionine are not involved in reaction (b) is indicated by the inability of these compounds to reverse selenate. This is further substantiated by their inability to further enhance the activity of methionine in a sulfate free medium. This is shown in Table XII.

The data further emphasize the inadequacy of cystine and glutathionine as a source of sulfur for yeast. In addition, evidence is at hand that both these substances can be synthesized from methionine. The growth obtained with methionine as the sulfur source has been found to equal that obtained from sulfate. This is illustrated in Table XIII which gives the results of nine experiments in which a comparative concentration of sulfate and methionine has been used.* This is also illustrated by the ability of yeast to synthesize thiamin equally from sulfate or methionine (Table XIV). Although it has been found that thiamin is capable of enhancing the effect of methionine in selenate inhibition (Table XV), the observation that thiamin can be synthesized equally from either

* The assumption was made initially that only the L-form of methionine could be utilized, so that growth obtained from 2 mg. DL-methionine per tube, which represents 0.21 mg. available sulfur, could be compared with growth from 2.5 mg. $\text{Na}_2\text{SO}_4 \cdot 10\text{H}_2\text{O}$ which represents 0.24 mg. available sulfur. Later in this section it will be shown that D-methionine can be utilized to some extent so that the available sulfur in 2 mg. DL-methionine is actually greater than 0.21 mg. Nevertheless, the growth in DL-methionine and sulfate is equivalent.

Table XII.

The Action of Cystine and Glutathione in Conjunction
With Methionine in Selenate Toxicity in Yeast

<u>H₂SeO₄</u> mg.	<u>Methionine</u> mg.	<u>Cystine</u> mg.	<u>Glutathione</u> mg.	<u>Optical Density</u>
0	0	0	0	0.15
0	2	0	0	0.88
1	2	0	0	0.61
0	0	2	0	0.21
1	0	2	0	0.00
0	0	0	2	0.20
1	0	0	2	0.00
0	2	2	0	0.81
1	2	2	0	0.57
0	2	2	2	0.88
1	2	2	2	0.55

Incubation time: 48 hours

Table XIII.

The Comparison of Yeast Growth in Sulfate and Methionine#

<u>Na₂SO₄·10H₂O</u>	<u>DL-methionine</u>	<u>Incubation</u>
<u>0.d.*</u>	<u>0.d.*</u>	<u>hours</u>
0.72	0.70	43
0.96	0.88	48
1.25	1.25	67
0.69	0.68	36
0.99	0.98	120
1.02	1.01	120
0.98	1.01	120
1.08	1.00	42.5
0.98	0.98	42.5

2.5 mg. of Na₂SO₄·10H₂O and 2 mg. DL-methionine per tube were used respectively.

* Optical density

Table XIV.

The Influence of Thiamin on Yeast Growth in the Presence of Sulfate and Methionine

<u>Na₂SO₄·10H₂O</u> mg.	<u>Methionine</u> mg.	<u>Thiamin</u> g.	<u>Optical Density</u>
0	0	0	0.13
2.5	0	0	0.51
2.5	0	0.01	0.56
2.5	0	0.05	0.60
2.5	0	0.10	0.67
2.5	0	0.50	0.70
0	2	0	0.53
0	2	0.01	0.52
0	2	0.05	0.55
0	2	0.10	0.62
0	2	0.50	0.76

Incubation time: 24 hours

Table XV.

The Effect of Thiamin Upon the Detoxication of
Selenate by Methionine#

<u>H₂SeO₄</u> mg.	<u>Thiamin</u> g.	<u>Optical Density</u>
0	0	0.92
1	0	0.72
1	0.5	0.87
1	1.0	0.90
2	0	0.58
2	0.5	0.71
2	1.0	0.71
3	0	0.46
3	0.5	0.61
3	1.0	0.61

Incubation time: 52 hours

#Basal medium used in which sulfate was replaced by
methionine (400 mg. per liter of double strength medium).

sulfate or methionine makes unnecessary the postulation as suggested in equation 2).

D. The Inactivation of Essential Cellular Components

The possibility exists that the mode of action of selenate as a toxic reagent for organisms rests on the oxidative capacity of the selenate. Methionine is known to be oxidized readily to the sulfoxide (88) or the sulfone (89) with various oxidizing agents. Melnikov (90) reports that organic sulfides are readily oxidized with SeO_2 to the sulfoxide or the sulfone depending upon the temperature and concentrations. Both the sulfoxide or sulfone have been shown to repress the growth of Lactobacillus arabinosus and Lactobacillus casei (91,92,93,94). Similarly, the rat has been shown incapable of substituting the sulfone for methionine in the diet (95).

If this is true for selenate then its action could be explained by its prevention of methionine utilization. An attempt was therefore made to oxidize methionine with selenic acid. It was found that methionine underwent a ready reaction with selenic acid at room temperature with the rate depending upon the concentrations of the starting materials and the temperature employed. By varying either the concentration or the temperature or means of isolation, crystalline substances were obtained which from their

physical aspects appeared to be different compounds. Melnikov (90) has emphasized the critical nature of experimental conditions in reactions with SeO_2 . The preparation of two such compounds will be described, although no attempt was made to characterize them in any detail.

Compound I-73

To three gm. of methionine were added 10 ml. of 87.2% H_2SeO_4 and 10 ml. H_2O . The reaction mixture immediately turned bright red then became dark purple. Much heat was evolved in the reaction and a garlic like odor became evident. The mixture was cooled under tap and allowed to stand for one hour at room temperature. The black precipitate was filtered through a medium glass fritted filter and the filtrate allowed to stand for an additional hour. Methyl alcohol (20 ml.) was added and the mixture cautiously made alkaline with piperidine (litmus), cooling under tap with each addition of piperidine. Acetone (250 ml.) was added and the mixture shaken several times. The piperidine layer was removed and placed in the refrigerator overnight. The resulting yellowish precipitate was washed several times with acetone until it appeared white. The compound gave a positive McCarthy-Sullivan test but unlike methionine it did not precipitate with HgCl_2 , CuSO_4 or picric acid, and

decomposed when dried in an air oven at 60°C . for ten minutes. The decomposed product turned reddish-brown and smelled like cooked cabbage.

The decomposed product was dissolved in a minimum of water, boiled on a steam bath and filtered. Four volumes of methyl alcohol were added and the mixture chilled for several hours. A white precipitate was obtained which was filtered, washed with acetone and air dried. No melting point was obtained up to 273°C ., although the compound melted quickly in a flame leaving a black residue. A sodium fusion followed by lead acetate gave a strong positive test for sulfur or selenium. The presence of selenium was indicated by a positive codeine test (96). A similar test with methionine was negative. The presence of sulfur was demonstrated in the following manner:

0.2 gms. of the compound were added to 50 ml. of fuming nitric acid in a porcelain crucible, and this heated (hood) on a waterbath to one half the original volume. Another 50 ml. of fuming HNO_3 were added and the heating continued until 2-3 ml. remained. This was allowed to cool and 10 ml. Br_2 in 40 ml. 48% HBr were added. This was heated gently until the excess Br_2 was distilled off, the waterbath was then exchanged for an asbestos pad and the mixture strongly heated until the volume was 2-3 ml. Upon cooling, a few drops of concentrated NaOH were

added to make the mixture alkaline, and the heating continued down to dryness. A sodium fusion was performed on the greyish residue and a positive test for sulfur obtained with lead acetate. To another aliquot of the residue, tests for selenium were negative.

Positive tests for nitrogen were obtained by the Lassaigne test utilizing the sodium fusion, as were fusion tests with soda lime. Amino nitrogen was indicated by reaction with HNO_2 with gas evolution. The compound did not precipitate with lead acetate, hence it is not a sulfide or selenide. Test for the thiol grouping was negative with nitroprusside. The compound was soluble in water or acidic and basic solutions, and only slightly soluble in methyl or ethyl alcohol (50%) from which it is best crystallized. Strong alkali did not produce any piperidine from it. When the reaction and isolation procedure was carried out without methionine, no product was obtainable.

Compound I-119

3 gms. SeO_2 were dissolved in 10 ml. H_2O and 3 gms. methionine added and dissolved. The mixture was placed on a waterbath and heated for 20 minutes. Upon cooling, long transparent needles separated out. This was separated from the elemental selenium by adding 5 ml. H_2O and

warming on a waterbath and filtering rapidly. To the filtrate was added acetone to a volume of 125 ml. A white precipitate formed immediately. The mixture was chilled for 1 hour. During this period the precipitate turned yellow-orange. It was collected on a filter, washed several times with acetone and air dried. The compound was recrystallized from hot acetone and water. Reddish, glistening crystals were obtained having an offensive odor. Weight: 2 gms.

In view of the different compounds that seemed possible between methionine and H_2SeO_4 or SeO_2 , depending upon the experimental conditions, no further work along this line was attempted.

When these compounds were tested as sources of sulfur for yeast, it was found that they were available to the organism in this capacity. However, they could not be used equally to reverse selenate inhibition (Table XVI). This was especially marked for Compound I-119 which was completely ineffective. Included in these experiments are compounds designated as I-80 and I-123 which have also been prepared from methionine and H_2SeO_4 and SeO_2 respectively. Although there is not assurance of their being different from the two previously described, the observation that I-80 did not give a positive McCarthy-Sullivan test and that I-123 appeared physically different in

color, odor, and form, warranted their inclusion in the experiments.

Similar to Compound I-119, were methionine sulfone,* and D-methionine which could be used as sulfur sources but could not reverse the toxic effect of selenate (Table XVI). A discussion of this point will be reserved for Section VII.

In pursuance of the possibility that the toxic action of selenate might be due to its inactivation of vital cellular constituents, experiments were performed to study the effect of various vitamins upon the detoxication. In these experiments the basal medium (Table I) was used with the modification that sulfate was replaced by methionine (400 mg. per liter of double strength medium). It was found that only thiamin was capable of enhancing the effect of methionine. The results after 52 hours have already been given in Table XV. Other vitamins tested but giving negative results were biotin (0.05 μ g.), nicotinic acid (10 μ g.), pantothenic acid (10 μ g.), riboflavin (10 μ g.), pyridoxine (10 μ g.), PABA (10 μ g.), inositol (250 μ g.), and folic acid (10 μ g.). The quantities in parenthesis represent the highest concentrations used per tube.

*The methionine sulfoxide and sulfone used in this section were prepared by the oxidation of methionine with H_2O_2 according to the methods of Toennies and Kolb (88,89). Methionine sulfoxide: C-36.6 (Theoretical 36.4) H-6.88 (Theoretical 6.68): Methionine sulfone: C-33.62 (Theoretical 33.30) H-6.29 (Theoretical 6.11.) The author is grateful to R. Lindsay for the analytical determinations.

Larger quantities of thiamin (up to 10 μ g.) than those listed in Table XV did not influence the detoxication further. It is of interest to note that SeO₂ has been found capable of in vitro oxidation of thiamin to the physiologically inactive thiochrome (98).

Table XVI.

The Availability of Methionine Derivatives as Sources of Sulfur for Yeast and Their Ability To Reverse Selenate Toxicity

<u>H₂SeO₄</u> mg.	<u>Sulfur Source (2mg.)</u>	<u>Optical Density</u>
0	DL-methionine	1.1
0	D-methionine	0.75
0	DL-methionine sulfoxide	1.1
0	DL-methionine sulfone	0.71
0	Compound I-80	1.1
0	Compound I-73	1.0
0	Compound I-123	1.0
0	Compound I-119	0.87
1	DL-methionine	0.73
1	D-methionine	0.00
1	DL-methionine sulfoxide	0.54
1	DL-methionine sulfone	0.00
1	Compound I-80	0.45
1	Compound I-73	0.68
1	Compound I-123	0.70
1	Compound I-119	0.00

Incubation time: 40 hours

SECTION VI. The Reversal of Selenate Inhibition in
Escherichia coli*

In Section III it was shown that methionine, but not cysteine or glutathione, was capable of partially reversing the toxic effect of selenate upon yeast growth. On the other hand, evidence presented below demonstrates that for E. coli, cysteine and to some extent glutathione, but not methionine is utilized in nullifying the selenate effect.

Experimental

The organism used was Escherichia coli, obtained from this laboratory's stock cultures. It was transferred daily into Harris and Kohn's " SG " Medium (69) (Table XVII) since the organism does not grow abundantly when introduced to this medium for the first time. As in the yeast experiments, a double strength medium ("SG") was prepared. All tubes received 5 ml. of medium and the total volume was increased to 10 ml. with distilled water after the various supplements were added. The inoculum was prepared by centrifuging a 24 hour culture of E. coli in " SG " medium and washing it twice in sterile saline. It was then suspended in twice its original volume of sterile saline of this suspension served as inoculum. The

* The contents of this section appeared in Archives of Biochemistry. (in press)

inoculated tubes were incubated at 37° C. Table XVIII illustrates the relative effectiveness of cysteine, glutathione and methionine to counteract the toxicity of selenate. The substances are indicated as supplements added per tube. Comparison with control tubes containing no selenate could not be made inasmuch as growth in selenate was accompanied by a red coloration.

In Section IV it was observed that 2-chloro-4-amino-benzoic acid which is considered a specific inhibitor for methionine synthesis in E. coli (70,73,74) had no action against Sacc. cerevisiae in a medium containing sulfate as the sole source of sulfur. In addition, it has been reported that cysteine but not methionine can serve as a satisfactory sulfur source for E. coli (99). In the present experiments, the reverse has been found true for Sacc. cerevisiae (Table XII). Schultz and Pomper (100) have recently shown that cystine could not be utilized for growth by forty members of the Genus Saccharomyces, including Saccharomyces cerevisiae, whereas methionine could be utilized by thirty six of the forty tested. It would appear from these observations, in addition to the data presented here, that the synthesis of methionine from sulfate is accomplished by different routes in these two organisms.

Table XVII.

Harris and Kohn's " SG " Medium-(Double Strength)

Glucose, anhydrous	3.2 gms.
MgSO ₄ ·7H ₂ O	0.32 gms.
KH ₂ PO ₄	3.2 gms.
(NH ₄) ₂ HPO ₄	3.2 gms.
NaCl	6.4 gms.
Tap H ₂ O	400 ml.
pH 7.2 with dilute NaOH and filter	

Table XVIII.

The Relative Effectiveness of Cysteine, Glutathione,
and Methionine Against Selenate Toxicity in E. coli

<u>H₂SeO₄</u> mg.	<u>Cysteine</u> mg.	<u>Glutathione</u> mg.	<u>Methionine</u> mg.	<u>Optical Density</u>
1	0	0	0	0.00
1	0.5	0	0	0.83
1	1.0	0	0	0.86
1	2.0	0	0	0.89
1	0	1.0	0	0.50
1	0	2.0	0	0.53
1	0	0	1.0	0.00
1	0	0	2.0	0.00
1	0	0	4.0	0.00
3	0	0	0	0.00
3	0.5	0	0	0.58
3	1.0	0	0	0.80
3	2.0	0	0	0.80
3	0	1.0	0	0.08
3	0	2.0	0	0.15
3	0	0	1.0	0.00
3	0	0	2.0	0.00
3	0	0	4.0	0.00

Incubation time: 52 hours

SECTION VII. Discussion and Critique

It would appear from the evidence thus far presented that previous proposals to explain the toxicity of selenium compounds, while they might be useful and applicable in animals, have no experimental support in yeast. An explanation of the toxicity in terms of glutathione is at present unsatisfactory. This is exemplified by its inability to influence the reversal of selenate toxicity in yeast. Although the administration of glutathione has reduced the mortality and toxic symptoms due to selenium salts in animals, this has only been the case when the compound was given simultaneously with the selenium salt (45,66,97).

The inhibition of animal dehydrogenases by selenite, although an experimentally established fact, is not observed with selenate in concentrations which are normally toxic to animals (21). Although this in itself is not necessarily contrary to the hypothesis that selenium toxicity is due to this inhibition, since the results were obtained from in vitro experiments, still, one would anticipate that anaerobic organisms which obtain their energy primarily by means of dehydrogenases, would be considerably more sensitive toward selenium reagents than are aerobic forms. This has not been found to be the case (41).

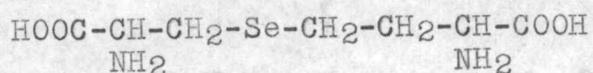
In the case of yeast which cannot utilize succinic acid as a source of carbon,* it is evident that succinic dehydrogenase is not involved in the toxic reaction. Furthermore whereas it is possible that the toxic action of selenate and selenite might be due to their oxidizing capacities, the same explanation could hardly hold for selenocystine, diselenodiacetic acid or other toxic organic selenium compounds (101,102). It can be inferred from this that not all selenium compounds have a similar mode of action, nor need they be delegated to one system. This is demonstrated by the comparative toxicity of inorganic and organic selenium compounds. Franke and Painter (103) found that there was little difference in the toxicity of selenium in various cereals, but these were definitely more toxic than inorganic salts. In spite of the fact that synthetic organic selenium compounds are less toxic than selenite, they produced similar symptoms of poisoning (102). The selenium of toxic grains which is generally considered to be organic (104,105,106) is more toxic than selenite (103,107,108).

The proposal of Moxon and Franke (24) that any compound capable of forming H_2S would detoxify selenium

* Twenty strains of Saccharomyces cerevisiae were inoculated into a routine synthetic medium (53) in which sucrose was replaced by succinate on an equal molar basis. These failed to grow whereas controls utilizing sucrose grew normally.

salts has not found support in the present experiments. This has been demonstrated by the absence of a precipitate in the Biginelli's solution in the methylation experiments. Any H₂S formed would have been carried over into the gas washing bottles and precipitated as HgS.

In the opinion of the present author, the toxic effect of selenate in yeast is a multiple one in which more than one system is attacked simultaneously. Two of these undoubtedly involve methionine and thiamin. With methionine, the toxicity appears due to the prevention of its utilization, the alteration of methionine residues in enzyme systems, or both. It is interesting to note in this connection that both thiamin and methionine contain the thio ether linkage. An important finding which deserves consideration at this point is the isolation of a selenium containing amino acid from toxic plants by Horn and Jones (106). The structure of the compound as suggested by the empirical formula is envisaged by the authors as



occurring in mixed crystals with its sulfur analog. This substance will immediately be recognized as the selenium analog of cystathione, which is at present considered to be an intermediate in the formation of cysteine from

methionine in animals (109) and the formation of methionine from cysteine in *Neurospora* (110). If the formation of this selenium compound is of general occurrence, then the interference of methionine utilization in yeast and cysteine utilization in *E. coli* (see Section VI) can be understood readily.

Any explanation of the role of methionine in reversing the toxicity of selenate must involve the specificity of the reaction. In particular, it is necessary to account for the ability of yeast to utilize D-methionine and methionine sulfone as sulfur sources but not as agents which can reverse the effect of selenate. On the other hand, L-methionine can function in both these capacities. Although there is no evidence available which can be used in the formation of a hypothesis, it does not seem improbable that the determining factors for the reversal process are the reaction rates for the specific reactions involved. In this case it must be assumed that yeast normally converts D-methionine to the L-configuration before utilization and that the sulfone is not used as such but is converted to methionine. Evidence that this is probably true for the sulfoxide in animals has been furnished by Virtue and Doster-Virtue (111).

To explain the inactivity of D-methionine and the sulfone toward selenate, it must be further assumed that

the rate of their conversion to L-methionine is not sufficiently rapid to be of any influence before other systems are attacked. One of these systems undoubtedly involves thiamin which can be synthesized from methionine (Table XIV). The observation that a correlation exists between the oxidation state of DL-methionine sulfoxide as compared to DL-methionine and DL-methionine sulfone and the extent of reversal of selenate by the sulfoxide as compared to DL-methionine and the sulfone is in accordance with this reasoning.

The Formation of Elemental Selenium

The appearance of an orange-red coloration accompanying growth in the presence of selenium salts has been often recorded in the literature, and has been observed by the present author in all experiments of long duration. It has been noted as early as 1890 by Chabrié and Lapique (27) in yeast. Subsequent to this, it has been observed in numerous bacteria (41,20). In all cases, it has been assumed to be elemental selenium although no actual proof of this has been found by the present author. The substance is intracellular and cannot be extracted out, although it can be decolorized by washing with KCN (25). Decolorization by the sodium polysulfide has also been found possible by the present author which suggest that

the substance might indeed be elemental selenium. The present author has found that it is possible to obtain the reddish coloration in absence of any nutrient substrate. By incubating a washed yeast (2 gms.) suspension in 250 ml. of phosphate buffer in the presence of 128 mg. of selenate, reddish cells were obtained within 48 hours which could not be decolorized by washing. The intensity of the color increased with longer incubation. It would thus appear that the formation of this substance is independent of the exogenous metabolism and probably involves a cellular component. Whether this is a selenate decomposing enzyme as postulated by Rosenfeld and Beath (17), cannot be stated with certainty. The existence of many cellular compounds that are capable of reacting with selenate makes it difficult to limit the explanation in terms of enzymatic activity. Likewise the supposition that yeast contains an enzyme which specifically decomposes selenate does not seem reasonable in the light of the multifarious activity of this organism toward "unnatural" and synthetic compounds (112).

However, regardless of how the substance is formed, its importance to the toxicological mechanism is at once apparent. The insolubility and non-diffusibility of the substance, plus the observation that the cell cannot eliminate it by metabolic means account for its intra-

cellular accumulation. This could only lead to the obstruction of normal functioning and finally to cellular destruction. It accounts for the observation of Potter and Elvehjem (19) that selenium salts become more toxic with time. It also is in harmony with the findings of Franke (113) that selenized animals never completely recover from the toxic effects. Finally, it can very well account for the toxic nature of selenium compounds in general. Shown in Plate I are unstained cells which were placed in the basal medium containing methionine instead of sulfate. Plates II and III illustrate the intracellular deposition of the insoluble red substance (Se?) at the end of five and ten days respectively. The extracellular granules in Plate III are the result of cellular breakdown. All cells are unstained.

SUMMARY

1. Inhibition of yeast growth by selenate is found to be reversed by the addition of methionine. No other organic compound tested displayed this activity. Within limits, the growth of yeast in the presence of selenate is directly proportional to the methionine present.

2. A methionine free casein hydrolysate was prepared and found to have no reversing effect. The addition of DL-methionine renders it active.

3. Normal casein hydrolysate is capable of partially reversing the selenate inhibition. The addition of methionine enhances this activity.

4. Yeast cannot utilize DL-homocystine plus a methyl donor such as choline, betaine, or creatinine to reverse the inhibition. This suggests that methionine is synthesized in a manner different from that in mammalian tissue.

5. Of the optical isomers of methionine, only the naturally occurring L-form is active.

6. A scheme for the synthesis of methionine from sulfate in yeast is suggested. It is substantiated by the behavior of sulfate, methionine and PABA in selenate and sulfonamide inhibition.

7. A relatively constant ratio has been found for a given concentration of selenate causing inhibition,

and for the amount of sulfate necessary to reverse it. This suggests that the mode of action of selenate toxicity in yeast is a competitive inhibition between sulfate and selenate resulting in the blocking of methionine synthesis. This is operative when sulfate is the sole source of sulfur.

8. No inhibition has been observed in yeast with 2-chloro-4-aminobenzoic acid, contrary to results previously obtained with E. coli.

9. The role of methionine in reversing selenate toxicity has been investigated. Evidence is presented which makes it improbable that the detoxication occurs via an oxidation of methionine to sulfate.

10. Evidence is presented against a detoxication mechanism involving methylation by methionine. This consists of the failure to isolate dimethyl selenide under aerobic and anaerobic conditions, the inability of methionine to influence inhibition by quinoline, the lack of inhibition by nicotinic acid, nicotinamide and pyridine and finally the observation cited in item 4 of this summary.

11. Equal growth is obtained from methionine as compared to sulfate. This indicates that the same compounds can be synthesized from both these substances.

12. Thiamin has been found to be the only substance of those tested which is capable of enhancing the reversal

of selenate by methionine.

13. Methionine has been found to react readily with H_2SeO_4 or SeO_2 . Crystalline compounds containing Se, N, and S have been isolated and their preparation is given.

14. D-methionine and DL-methionine sulfone have been found available to yeast as sulfur sources but incapable of affecting selenate reversal.

15. Cysteine, and to some extent glutathione, is effective in counteracting the inhibiting effect of selenate in E. coli. Methionine does not exhibit such action.

16. Evidence is presented which indicates that the synthesis of methionine from sulfate is accomplished by different routes in E. coli and Saccharomyces cerevisiae.

17. The opinion is expressed that the toxicity of selenium compounds is a multiple one in which several systems are attacked simultaneously. Two of these undoubtedly involve methionine and thiamin and their utilization. A third toxic effect is the intracellular accumulation of an insoluble, non-diffusible substance which is probably elemental selenium. This is demonstrated photographically.

18. A discussion is given which attempts to explain the specificity of methionine action.



Plate I. Unstained Yeast Inoculum Cells

Magnification: About 1200X

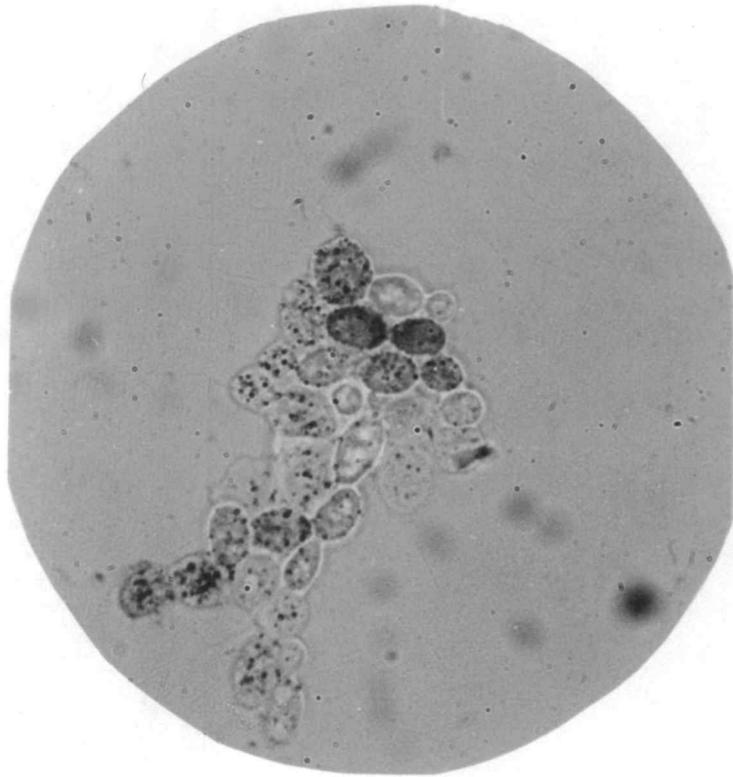


Plate II. Unstained Yeast Cells Grown
in the Presence of Selenate

Incubation: 5 days

Magnification: About 1200X

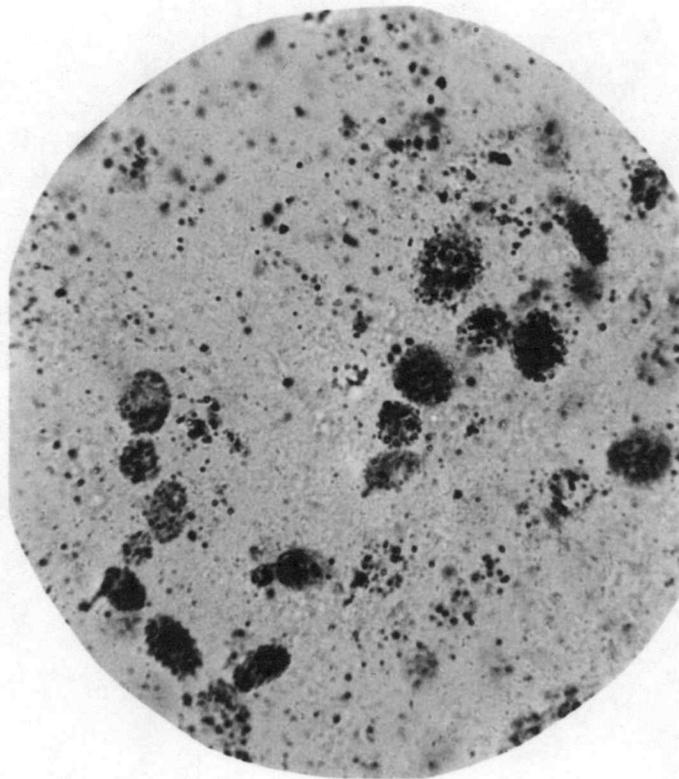


Plate III. Unstained Yeast Cells Grown
in the Presence of Selenate

Incubation: 10 days

Magnification: about 1200X

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