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# Effects of Molybdate, Tungstate, and Selenium Compounds on Formate Dehydrogenase and Other Enzyme Systems in *Escherichia coli*[1]

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The role of selenium and molybdenum in the metabolism of *Escherichia coli* was explored by growing cells in a simple salts medium and examining the metabolic consequences of altering the concentration of molybdenum and selenium compounds in the medium. The addition of tungstate increased the molybdate deficiency of this medium, as reflected by lowered levels of enzyme systems previously recognized to require compounds of molybdenum and selenium for their formation [formate-dependent oxygen reduction, formate dehydrogenase (FDH) (EC 1.2.2.1), and nitrate reductase (EC 1.9.6.1)]. The requirement for selenium and molybdenum appears to be unique to the enzymes of formate and nitrate metabolism since molybdate- and selenite-deficient medium had no effect on the level of several dehydrogenase and oxidase systems, for which the electron donors were reduced nicotinamide adenine dinucleotide, succinate, D- or L-lactate, and glycerol. In addition, no effect was observed on the growth rate or cell yield with any carbon source tested (glucose, glycerol, DL-lactate, acetate, succinate, and L-malate) when the medium was deficient in molybdenum and selenium. DL-Selenocystine was about as effective as selenite in stimulating the formation of formate dehydrogenase, whereas DL-selenomethionine was only 1% as effective. In aerobic cells, an amount of FDH was formed such that 3,200 or 3,800 moles of formate were oxidized per min per mole of added selenium (added as DL-selenocystine or selenite, respectively).

Selenium has been implicated as an essential trace element in the nutrition of animals and some species of plants. Schwarz (23) has found that selenite and an as yet uncharacterized organic selenium compound from natural sources (factor-3) are effective in preventing experimental nutritional liver diseases and death in rats. Thompson and Scott (30) have shown that a diet deficient only in selenium is fatal in chicks. Oldfield et al. (16) have shown that dietary selenium prevents white muscle disease in sheep. Trelease and Trelease (31) reported that selenium is required for normal growth and development in the selenium indicator species of *Astragalus* (vetch). However, neither the molecular basis of selenium requirement nor its functional form is known. One theory holds that this element functions in the cell as an antioxidant (28, 29), but Schwarz (23) concludes that this is not sufficient in explaining many of the physiological characteristics of selenium deficiency.

The biological fate of selenium is not well understood. Selenite is known to be incorporated into the proteins of plants, animals, and microorganisms as selenium analogs of the sulfur amino acids (2, 14, 22, 33, 34); however, seleno-amino acids per se have not been ascribed an essential function in a specific protein. Little work has been done to determine whether selenium is an essential micronutrient in bacteria. Although it was known as early as 1954 that selenite is required for the formation of formate dehydrogenase (FDH) in *Escherichia coli* (21), it is still not known what its function is in the formation of this enzyme or if it is required for the formation of any other *E. coli* enzymes.

Molybdenum-containing enzymes have been found in a number of bacteria, including the nitrate reductase of *E. coli* (27). It is known

[1] This work represents part of a doctoral dissertation to be submitted by H.G.E. to the Dept. of Biochemistry, College of Medicine, Univ. of Kentucky.

that molybdate is also required for the formation of FDH and cytochrome $b_{555}$ in *E. coli* grown anaerobically with nitrate, as well as for formate oxidase and formate hydrogenlyase of aerobic and anaerobic cells (7, 11, 21). It has not been determined if FDH contains molybdenum or if molybdate plays some other role in the formation of this enzyme. A number of workers have reported that tungstate is a competitive inhibitor of molybdenum utilization in plants and bacteria (8, 15, 26). Tungstate has also been shown to antagonize the formation of the FDH of *E. coli* grown anaerobically with nitrate (21).

In this study, we attempted to find (i) whether tungstate could inhibit the formation of known molybdate-dependent enzymes in *E. coli*, (ii) whether a requirement for selenite or molybdate could be demonstrated for any other *E. coli* enzymes, with special reference to the membrane-bound dehydrogenase and oxidase systems, and (iii) whether some form of selenium other than selenite could stimulate the formation of FDH.

## MATERIALS AND METHODS

**Culture methods.** *E. coli* HfrH (*thi*$^-$) used in all experiments was obtained from J. A. DeMoss. Anaerobic growth conditions on minimal salts medium with nitrate were described previously (11).

For aerobic growth, the same medium was employed except that $KNO_3$ and $KHCO_3$ were omitted (initial *p*H: 7.1 to 7.2) and the carbon source was 0.5% glucose (w/v) unless otherwise noted. All cultures were inoculated with cells grown on selenium- and molybdenum-deficient medium. Cultures were incubated in Erlenmeyer flasks at 37 C on a gyrotory incubator shaker (model G-25 New Brunswick). Unless otherwise noted, $Na_2MoO_4$, $Na_2SeO_3$, and $Na_2WO_4$ when added were autoclaved in the medium. Growth was estimated turbidimetrically by measuring the optical density at 600 nm ($A_{600}$) with a spectrophotometer (Spectronic 20, Bausch & Lomb). Samples were diluted to read below $A_{600}$ = 0.5. Cells for enzyme analysis were harvested by centrifugation in the cold, washed twice with 50 mM sodium phosphate (*p*H 7.2), and resuspended in this buffer to a concentration of 5 to 20 mg of protein per ml. Samples were stored at 0 C since it was found that freezing destroyed over 50% of the FDH activity. Assays of whole cells were performed within 1 day of harvest, and assays on the membrane fraction were performed within 5 days of harvest. Cell protein was estimated by the method of Lowry et al. (13) as described previously (11).

**Enzyme assay procedures.** All assays were performed at 30 C, and in each case it was determined that the rate was proportional to enzyme concentration.

Reduced benzyl viologen:nitrate reductase (NR), reduced benzyl viologen:chlorate reductase, and formate dehydrogenase [dichlorophenolindophenol (DCPI) reduction mediated by phenazine methosulfate (PMS)] were measured as previously described (11).

NADH dehydrogenase (reduced nicotinamide adenine dinucleotide:ferricyanide oxidoreductase, EC 1.6.99.2) was measured by following the anaerobic reduction of ferricyanide at 420 nm with a Cary 15 recording spectrophotometer. The reaction mixture of 4 ml contained 0.16 mM $K_3Fe(CN)_6$ and 50 mM sodium phosphate (*p*H 7.2). NADH (final concentration 0.2 mM) was added, and the endogenous rate was followed for several minutes. The reaction was started by the addition of enzyme. The rate was extrapolated from initial velocity, and for final calculations an extinction coefficient of 1.0 $mM^{-1}$ $cm^{-1}$ was used.

Succinic dehydrogenase (succinate:PMS oxidoreductase, EC 1.3.99.1) was measured as suggested by Singer and Kearney (24) by following the anaerobic reduction of DCPI mediated by PMS at 600 nm. A reaction mixture of 4 ml contained 0.05 ml of 6 mM DCPI (0.075 mM), 2 mM PMS, 2 mM KCN (freshly prepared), and 50 mM sodium phosphate (*p*H 7.8). Due to the high concentration of PMS required there was a non-enzymatic rate of DCPI reduction which was followed for several minutes. Enzyme was added, and the endogenous rate was measured. Neutralized sodium succinate (final concentration of 12 mM) was added, and the final rate was measured. The reaction became linear after 3 to 4 min. The final velocity was determined by subtracting the endogenous and non-enzymatic rates from the total rate. An extinction coefficient of 21 $mM^{-1}$ $cm^{-1}$ was used.

D-Lactic and L-lactic dehydrogenases were also measured by following the anaerobic reduction of DCPI with PMS. The reaction mixture of 4 ml contained 0.075 mM DCPI, 0.25 mM PMS, and 50 mM tris(hydroxymethyl)aminomethane (Tris) buffer (*p*H 7.2). Enzyme was added, and endogenous rate was measured. Neutralized sodium D-(−)-lactate or sodium L-(+)-lactate (final concentration of 10 mM or 20 mM, respectively) was added, and the final rate was measured. The rate was linear for at least 5 min.

Oxidase activities were measured polarographically with a Clark-type oxygen electrode (model 53, Yellow Springs Instrument Co.). All assays were in 50 mM sodium phosphate (*p*H 7.2) in a total volume of 4 ml. In each case, enzyme was added and the endogenous rate recorded, then substrate was added and the final rate measured. Final concentrations of substrates (neutralized with NaOH) were 20 mM formate, 1.0 mM NADH, 10 mM D-(−)-lactate, 20 mM L-(+)-lactate, or 25 mM glycerol. All rates were linear for at least 5 min. Calculation of specific activities was based on an oxygen concentration of 0.235 mM for saturated buffer at 30 C and 1 atom of oxygen.

Preparation of membrane fraction. Cell envelope was prepared by a modification of the lysozyme-ethylenediaminetetraacetic acid (EDTA) method of Kundig and Roseman (10). Frozen-thawed cells were washed once with 10 mM Tris buffer (*p*H 8.0) which contained 0.2 mM dithiothreitol, resuspended in 40 ml of the same buffer at 4 mg of protein per ml, and

allowed to come to room temperature. EDTA (0.8 ml of 0.25 M, *p*H 8.0) was added, and the mixture was stirred for 20 min. Then 1.4 ml of 2 mg of lysozyme/ml (Worthington Biochemicals) was added. After stirring for 45 min, 0.4 ml of 1.0 M $MgSO_4$ and 0.4 ml of 1.0 mg of bovine deoxyribonuclease I (Worthington Biochemicals) per ml was added, and mixture was stirred for another 10 min. Finally, EDTA (1.07 ml of 0.25 M, *p*H 8.0) was added. Up to this point (from the time the cells were warmed to room temperature) the preparation was kept under an atmosphere of argon. The mixture was frozen at −17 C; after 30 min it was thawed and centrifuged in the cold for 15 min at 10,000 × *g*. The pellet was washed twice with 50 mM sodium phosphate (*p*H 7.2) and resuspended in the same buffer at a protein concentration of 10 to 20 mg/ml. This was called the membrane fraction; it was stored at 0 C and assayed within 24 hr of preparation. This method typically yielded 70 to 80% solubilization of cellular protein and recovery of more than 90% of FDH activity.

Construction and operation of anaerobic cuvette. A cuvette of simple construction using cheap materials has been used here and previously (11) in anaerobic assays. The body of the cuvette was made by joining a length of 10-mm internal diameter square tubing (Ace Glass) to a standard taper (24/25) ground-glass joint and sealing the bottom. The plug was made from a Teflon or polyvinyl chloride bar tapered to give a gas-tight fit requiring no lubricant. A hole was drilled through the center of the plug to accomodate a length of heavy wall polyethylene tubing (0.042-inch internal diameter, 0.114-inch outside diameter, Bel-Art, no. F-21852) which serves as the gas inlet line (A in Fig. 1). The fit here should be snug but still allow the tubing to slide up and down with application of some moderate force. Another hole for gas exit and sample port (B in Fig. 1) was drilled at an angle such that it crossed the centerline about 5 mm below the plug. A 0.042-inch diameter hole is just large enough to accommodate a 100-μliter syringe (C in Fig. 1) without completely plugging the hole. The sample holder (D in Fig. 1) was made by cutting a 4-mm length of 0.25-inch outside diameter Teflon tubing, flattening it on the side with a hammer, and slipping it on to the end of the gas inlet line.

In operation, the gas inlet line is lowered so that the assay mixture is deoxygenated by bubbling with purified argon (11) for 2 min at a flow rate of about 100 ml/min. The gas inlet line is raised, and sample additions are made with a microsyringe inserted at B. The sample (50 μliters or less) is ejected into the sample holder and may be degassed by allowing it to equilibrate for several minutes in the argon atmosphere before plunging the gas inlet line into the assay solution where mixing is rapidly accomplished by the bubbling argon. During the course of the assay argon is blown over the surface of the solution. The oxidation of reduced benzyl viologen ($A_{600}$ = 2.0) in this system was very slow ($\Delta A_{600} < 0.002$ per min). Another effective cuvette for anaerobic assays has been recently described by Dixon (6). It is similar in that it allows the inert gas to constantly flush the system and reagents may be added to the assay mixture. However, in Dixon's device, since gas is bubbling into the solution during the course of the assay, the design must be considerably more demanding in its geometry so as not to disturb the light path.

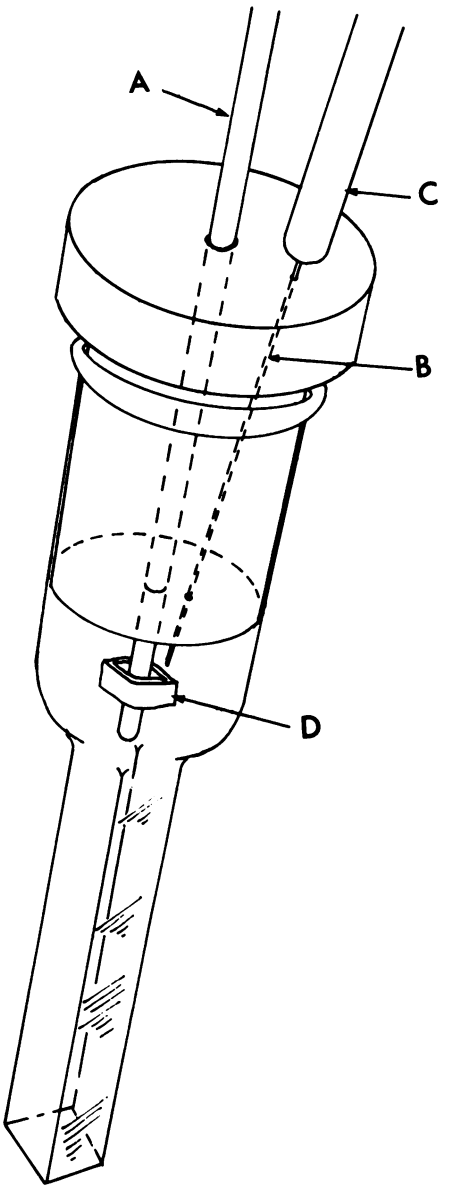


FIG. 1. *Apparatus used in anaerobic assays. A: gas inlet line; B: gas exit and sample port; C: microsyringe; D: sample holder.*

**Source of chemicals.** Chemicals were purchased from Sigma [DL-selenomethionine, D-(−)- and L-(+)-lactic acids), Calbiochem (DL-selenocystine), Fisher (sodium acetate and $Na_2WO_4$), Mallinckrodt (suc-

cinic acid), Eastman (L-malate), Baker & Adamson (95% glycerol), and others as previously described (11).

## RESULTS

Other than judicious use of reagent grade chemicals, no special precautions were taken in our experiments to eliminate molybdenum and selenium from the basal mineral salts medium. The growth medium requires added selenite plus molybdate to produce maximal levels of FDH (11, 21). Preliminary experiments indicated that the aerobic growth medium supplemented only with $10^{-7}$ M molybdate gave less than 5% of the maximal level of FDH, whereas medium supplemented only with $10^{-7}$ M selenite gave from 20 to 40% of the maximal level of FDH. Thus, judging from this response of FDH, we concluded that the unsupplemented medium was fairly free of selenite but contained a significant amount of molybdate. We have observed here and in previous studies (11) that when cells were grown anaerobically with nitrate with $10^{-6}$ M selenite, FDH activity was about 3% of the level which could be achieved when both selenite and molybdate were added. The FDH specific activity of anaerobically grown cells is about three to five times higher than that of aerobically grown cells. Addition of tungstate to this medium appears to reduce the effective molybdenum concentration. It can be seen in Table 1 that tungstate in $10^2$ to $10^4$ molar excess of added molybdate inhibits the formation of NR, a reported molybdenum-containing enzyme in *E. coli* (27). At the highest level of tungstate tested, the inhibition of NR is 54%, whereas for FDH it is 96%. It was thought possible that the NR activity remaining at high levels of tungstate might be due to another type of NR, for example, a non-molybdenum enzyme, Pichinoty (19, 20) has demonstrated a second type of NR, nitrate reductase B, in a number of enteric bacteria. In contrast to the nitrate-induced NR (A-type), NR B does not reduce chlorate and is much less sensitive to the inhibitor azide ($K_i > 10^{-4}$ M). However, $5 \times 10^{-5}$ M azide caused a 65% inhibition of the NR activities of cells grown on medium supplemented with either $10^{-7}$ M molybdate plus $10^{-3}$ M tungstate or with only $10^{-7}$ M molybdate. The ratio of reduced benzyl viologen-chlorate reductase:NR ranged from 1.0 to 1.4 in cells grown on medium supplemented with $10^{-7}$ M molybdate or $10^{-3}$ M tungstate, or both. This suggests that the bulk of the activity is due to NR A-type enzyme. That the inhibition of the formation of NR and FDH is due to a tungstate-molybdate competition is shown by the fact that at the highest level of tungstate ($10^{-3}$ M) normal levels of FDH and somewhat higher levels of NR are achieved if the molybdate concentration is raised to $10^{-4}$ M. The significance of the somewhat higher restored NR level has not been pursued. It is of interest to note that in the absence of added molybdate, $10^{-3}$ M tungstate caused a severe inhibition of anaerobic growth. It can be seen that molybdate is also effective in reversing this growth inhibition. When added directly to the assay mixture, $10^{-3}$ M tungstate had no effect on NR, FDH, or formate oxidase activities.

We have also found that tungstate inhibits the formation of FDH in aerobically grown *E. coli*. The inhibition is 94% with a $10^5$ molar excess of tungstate to added molybdate (Table

TABLE 1. *Effect of anaerobic growth medium supplements on the levels of formate dehydrogenase and nitrate reductase*[a]

| Expt | Supplements to the growth medium (M) | | | Doubling time (min) | $A_{600}$ at harvest | Specific activity | |
|---|---|---|---|---|---|---|---|
| | $Na_2SeO_3$ | $Na_2MoO_4$ | $Na_2WO_4$ | | | FDH | NR |
| 1 | | | | | 1.85 | <10 | 280 |
| | $10^{-6}$ | $10^{-7}$ | | | 1.40 | 1,630 | 1,550 |
| | $10^{-6}$ | $10^{-7}$ | $10^{-5}$ | | 1.44 | 590 | 960 |
| | $10^{-6}$ | $10^{-7}$ | $10^{-4}$ | | 1.05 | 260 | 750 |
| 2 | $10^{-6}$ | | $10^{-3}$ | >185 | 0.095 | <50 | 160 |
| | $10^{-6}$ | $10^{-7}$ | | 95 | 1.20 | 2,610 | 1,510 |
| | $10^{-6}$ | $10^{-7}$ | $10^{-3}$ | 135 | 0.78 | 110 | 700 |
| | $10^{-6}$ | $10^{-4}$ | $10^{-3}$ | 120 | 1.00 | 2,290 | 2,300 |

[a] Composition of the basal medium, anaerobic growth with nitrate, and the enzyme assays were those described in Materials and Methods. Cells from overnight cultures were harvested in exponential phase at the optical density ($A_{600}$) indicated. All assays were performed on whole cells. Specific activities are in nanomoles per minute per milligram of protein. FDH, formate dehydrogenase; NR, nitrate reductase.

2). In experiment 2, $10^{-4}$ M tungstate in the absence of molybdate reduced activity to 4% of that obtained with $10^{-6}$ M molybdate. The activity with tungstate alone was less than the activity with neither tungstate nor molybdate added. The effect of tungstate here is again reversed by higher levels of molybdate. Therefore, the addition of tungstate appears to reduce the effective molybdate concentration in aerobic cultures.

Using the criteria of absence of added selenite for selenite deficiency and the presence of $10^{-3}$ M tungstate in the absence of added molybdate for molybdate deficiency in the growth medium, we examined the effect of these growth conditions on some other oxidative enzymes in *E. coli*. It can be seen in Table 3 that the level of NADH dehydrogenase was unaffected in selenite- and molybdate-deficient medium, whereas the levels of FDH and formate oxidase decreased 180- and 48-fold, respectively, under these conditions. Table 4 shows that selenite and molybdate deficiency has no effect on the oxidation rate of D-lactic acid, L-lactic acid, or succinic acid of aerobically grown *E. coli* measured with $O_2$ or PMS as acceptor. Further evidence for the lack of involvement of selenite and molybdate in the stimulation of these (and other oxidative) enzymes comes from aerobic growth experiments

TABLE 2. *Effect of tungstate and molybdate on the formation of formate oxidase*[a]

| Expt | Supplements to the growth medium | | | Formate oxidase[b] |
|---|---|---|---|---|
| | $Na_2MoO_4$ (M) | $Na_2WO_4$ (M) | W/Mo | |
| 1 | | | 0 | 250 |
| | $10^{-7}$ | | 0 | 680 |
| | $10^{-8}$ | | 0 | 540 |
| | $10^{-8}$ | $10^{-4}$ | $10^4$ | 74 |
| | $10^{-7}$ | $10^{-3}$ | $10^4$ | 62 |
| | $10^{-8}$ | $10^{-3}$ | $10^5$ | 34 |
| 2 | | | 0 | 140 |
| | $10^{-6}$ | | 0 | 610 |
| | | $10^{-4}$ | | 22 |
| | $10^{-6}$ | $10^{-4}$ | $10^2$ | 670 |
| | $10^{-5}$ | $10^{-4}$ | 10 | 660 |
| | $10^{-4}$ | $10^{-4}$ | 1 | 620 |

[a] All cultures contained $10^{-6}$ M $Na_2SeO_3$ in addition to the normal aerobic growth medium. In experiment 1, cultures were grown for 12 hr and harvested at $A_{600}$ = 1.35 to 1.52; in experiment 2, cultures were grown for 10.5 hr and harvested at $A_{600}$ = 1.65 to 1.85. All assays were done on whole cells.

[b] Specific activity in nanomoles per minute per milligram of protein.

on various carbon sources (carbon source is growth limiting in these experiments). The absence of added selenite and molybdate from medium had no effect on the aerobic growth rate or cell yield (Table 5). The experiment in Table 6 suggests that neither selenite nor molybdate is involved in growth on glycerol or on the level of $O_2$ uptake with glycerol as substrate. The evidence suggests that the synthesis of the enzymes examined in this investigation either does not require selenium or molybdenum, or that only trace amounts are required.

We have tested the two selenium analogs of the sulfur-containing amino acids for the ability to stimulate FDH formation. DL-Selenocystine, DL-selenomethionine, or selenite was added with molybdate to cultures of *E. coli*, and the level of FDH activity (measured as formate oxidase activity units per ml) and growth (mg of cellular protein per ml) were measured during exponential growth. Figure 2 shows that DL-selenocystine (and possibly DL-selenomethionine) stimulate the same differential rate of FDH synthesis as selenite. At limiting concentrations of the selenium compounds, the level of FDH activity reaches a limit, from which the level of FDH activity per mole of selenium added can be calculated. We find that, per atom of selenium, DL-selenocystine and selenite are equally effective, whereas DL-selenomethionine is 1/100 as effective in stimulating FDH activity. If only the L-isomer of DL-selenocystine were utilized, then L-selenocystine would be about twice as effective as selenite. We have also found that L-cystine ($10^{-7}$ M) does not stimulate the formation of FDH.

We used paper chromatography (33, 34) to determine whether the organo-selenium compounds were contaminated with selenite. The solvent mixture used was isopropyl alcohol-formic acid-water (70:10:20; selenomethionine and selenocystine were located by ninhydrin spray, and selenite was detected by dipping the chromatogram in ethanol containing ascorbic acid which reduces selenite to red elemental selenium. We could not detect any selenite contamination, although we judge that 1 mole per cent could have been detected.

## DISCUSSION

It has been reported that tungstate antagonizes the molybdate stimulation of FDH in nitrate-grown but not in aerobically grown *E. coli* (21). We have shown that tungstate antagonizes the formation of NR in nitrate-grown

TABLE 3. *Effect of aerobic growth medium supplements on the formation of formate and NADH-oxidizing enzymes*[a]

| Supplements to the growth medium[b] | | | Oxidase activity[c] | | Dehydrogenase activity[d] | |
|---|---|---|---|---|---|---|
| $Na_2SeO_3$ ($10^{-6}$ M) | $Na_2MoO_4$ ($10^{-8}$ M) | $Na_2WO_4$ ($10^{-3}$ M) | Formate[e] | NADH[f] | Formate | NADH |
| − | − | − | 16 | 320 | <5 | 260 |
| + | − | − | 140 | 350 | 61 | 230 |
| − | + | − | 37 | 330 | 13 | 230 |
| + | + | − | 760 | 390 | 905 | 250 |
| + | − | + | 21 | 370 | <5 | 240 |
| + | + | + | 18 | 330 | <5 | 260 |

[a] Cells were harvested after 10 hr of growth at $A_{600}$ = 0.99 to 1.23. NADH, reduced nicotinamide adenine dinucleotide.

[b] Plus indicates that supplements were added; minus indicates that no supplements were added.

[c] Expressed as nanomoles per minute per milligram of protein.

[d] Assay done on membrane fractions. Expressed as nanomoles per minute per milligram of protein.

[e] Assay done on whole cells.

[f] Assay done on sonic extract. Just before assay, cells (20 to 30 mg of protein per ml) were sonically treated with four 5-sec pulses with the large tip of a Branson sonifier at the no. 8 intensity setting.

TABLE 4. *Effect of growth medium supplements on the level of Escherichia coli oxidative enzymes*[a]

| Supplements to the growth medium[b] | | | Oxidase specific activities | | | Dehydrogenase specific activities | | | |
|---|---|---|---|---|---|---|---|---|---|
| $Na_2SeO_3$ ($10^{-6}$ M) | $Na_2MoO_4$ ($10^{-8}$ M) | $Na_2WO_4$ ($10^{-3}$ M) | Formate | D-Lactate | L-Lactate | Formate | D-Lactate | L-Lactate | Succinate |
| − | − | − | <5 | 56 | 19 | <1 | 87 | 28 | 130 |
| + | + | − | 740 | 52 | 16 | 580 | 73 | 23 | 110 |
| + | − | + | <5 | 54 | 18 | <1 | 89 | 28 | 140 |

[a] Cultures were grown for 10 hr and harvested at $A_{600}$ = 1.41 to 1.53. All assays were done on membrane fractions and were described in Materials and Methods. Specific activities are in nanomoles per minute per milligram of protein.

[b] Plus indicates that supplements were added; minus indicates that no supplements were added.

TABLE 5. *Effect of metal supplements on the ability of Escherichia coli to grow on various carbon sources*[a]

| Carbon source | Doubling time (min) | | Cell yield ($A_{600}$) | |
|---|---|---|---|---|
| | +Mo, +Se[b] | −Mo, −Se[c] | +Mo, +Se[b] | −Mo, −Se[c] |
| Glucose | 75 | 75 | 1.05 | 1.08 |
| Sodium acetate | 200 | 195 | | |
| Succinic acid | 90 | 90 | 0.53 | 0.54 |
| L-Malic acid | 80 | 85 | .43 | .44 |
| DL-Lactic acid[d] | 105 | 105 | .40 | .41 |

[a] Cultures were grown on normal aerobic medium, except 0.5% glucose was replaced by the carbon source indicated at 0.1% (w/v). Initial *p*H of medium was 6.9 to 7.1.

[b] Medium contained $10^{-6}$ M $Na_2MoO_4$ and $10^{-6}$ M $Na_2SeO_3$.

[c] No $Na_2MoO_4$ or $Na_2SeO_3$ added to medium.

[d] DL-Lactic acid, 85% (Mallinckrodt).

cells and also, at higher levels, the FDH or formate oxidase of aerobic cells. In nitrate-grown cells, tungstate causes a greater inhibition of the formation of FDH than NR. This difference may be an indication that NR has a greater affinity for molybdate than FDH or that NR is more specific in its recognition of molybdate; however, a more complex explanation may be required.

It has been established that *E. coli* grown anaerobically with nitrate requires molybdate for the formation of NR and cytochrome $b_{555}$, and molybdate plus selenite for the formation of aerobic and anaerobic FDH (7, 11, 21). The enzyme systems regulated by selenite and molybdate, formate oxidase, formate hydrogenlyase, and nitrate reductase, involved membrane-bound electron transport enzymes. In this regard, it is noteworthy that Schwarz (23) has reported lowered respiratory activity in liver slices of rats raised on a selenium-de-

TABLE 6. *Effect of metal supplements on the ability to utilize glycerol as carbon source*[a]

| Supplements to the growth medium | | | Generation time (min) | Cell yield ($A_{600}$) | Oxygen uptake[b] with | |
|---|---|---|---|---|---|---|
| $Na_2SeO_3$ ($10^{-6}$ M) | $Na_2MoO_4$ ($10^{-8}$ M) | $Na_2WO_4$ ($10^{-3}$ M) | | | Formate | Glycerol |
| − | − | − | 90 | 1.39 | 14 | 290 |
| + | + | − | 90 | 1.38 | 520 | 280 |
| + | − | + | 90 | 1.35 | 7 | 280 |

[a] Cultures were grown in normal aerobic medium, except 0.5% glucose was replaced by 0.2% glycerol (w/v). Cells for oxidase assays were harvested after 12 hr of growth at $A_{600}$ = 1.02 to 1.24. Oxygen uptake with formate or glycerol as substrate was measured polarographically as described in Materials and Methods. Assays were done on whole cells. Plus indicates supplement added; minus indicates no supplement added.

[b] Expressed as nanoatoms of oxygen per minute per milligram of protein.

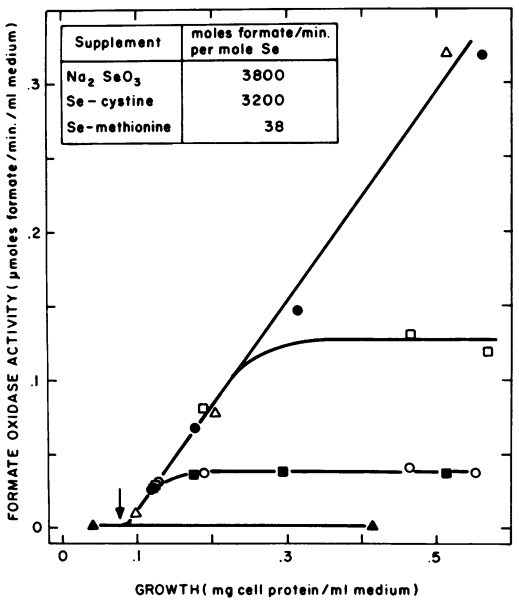


FIG. 2. *Synthesis of formate dehydrogenase in the presence of molybdate, and either selenite, selenocystine or selenomethionine during exponential aerobic growth. Cultures were inoculated with cells at approximately 0.03 mg of protein/ml. Enzyme yield was measured by the formate oxidase assay; growth was measured as described in Materials and Methods and converted to protein concentration (at $A_{600}$ = 1.0, the medium contains 0.32 mg of protein/ml). The arrow indicates the addition of $10^{-6}$ M $Na_2MoO_4$ and $10^{-7}$ M DL-selenocystine (△); $10^{-6}$ M $Na_2MoO_4$ and $10^{-7}$ M $Na_2SeO_3$ (●); $10^{-6}$ M $Na_2MoO_4$ and $10^{-8}$ M DL-selenocystine (□); $10^{-6}$ M $Na_2MoO_4$ and $10^{-8}$ M $Na_2SeO_3$ (○); $10^{-6}$ M $Na_2MoO_4$ and $10^{-6}$ M DL-selenomethionine (■); no addition (▲). The inset shows the formate oxidase activity per mole of selenium added at $10^{-7}$ M sodium selenite or selenocystine or $10^{-6}$ M selenomethionine.*

ficient diet. The NADH dehydrogenase of *Azotobacter vinelandii* grown to iron-deficient medium is reported to contain molybdenum (4). Conflicting reports exist concerning the possible role of molybdenum in the mammalian NADH oxidase chain. Albracht and Slater (1) reported the presence of an electron paramagnetic resonance signal at $g$ = 1.98 due to molybdenum and the presence of 1 mole of molybdenum per mole of flavine mononucleotide in Complex I, whereas Orme-Johnson et al. (17) reported the presence of this signal in submitochondrial particles and Complex I, which contain less than 0.03 atoms of molybdenum per acid-extractable flavin molecule. Our data suggest that neither selenium nor molybdenum is required for aerobic NADH oxidase or dehydrogenase activity of *E. coli*. The lack of effect of selenium or molybdenum deficiency on the oxidation of glycerol, D- and L-lactic acid, and succinic acid, or on growth rate or cell yield on glucose or a number of nonfermentable carbon sources suggests lack of involvement of selenium or molybdenum in the metabolism of these compounds. Of course we cannot completely exclude the possibility that selenium and molybdenum are required in the enzyme systems tested above; however, if a requirement exists it must be at a level much lower than that required for FDH.

Molybdate, selenite, and tungstate would appear to affect the formation of active enzymes rather than the permeability or transport of substrates. In the case of FDH and formate oxidase, these metal effects are seen with either intact- (Tables 1, 2, and 3) or broken-cell preparations (Tables 3 and 4), and as shown previously (21) enzyme activity did not increase upon cell breakage (Tables 2, 3, and 4). In other experiments, we have noted that whole- or broken-cell preparations had comparable NR activities which were dependent on the presence of molybdate in the anaerobic growth medium.

Since the effect of selenium in *E. coli* seems to be unique for FDH, it seems unlikely that

selenium functions as a general antioxidant in this bacterium as has been proposed for its role in higher organisms (28, 29). The FDH of *E. coli*, which under various growth conditions can provide electrons for $O_2$ reduction, nitrate reduction, or hydrogen production, remains the only definite enzyme in which a critical role can be attributed to selenium.

Since the mechanism of selenite action is not known, it would be helpful to know if some related compound could also stimulate the formation of FDH. Previous work has shown that selenate, tellurate, and tellurite cannot replace selenite (21). In seeking to find active forms of selenium, we tested several reduced selenium compounds for this ability. It was found that selenide added in the form of DL-selenocystine was as effective as selenite in this respect and is thus the only compound reported to be as effective as selenite. DL-selenomethionine, which was much less effective, is known to be able to fully replace methionine in a methionine-requiring mutant of *E. coli* (3). The fact that 3,200 moles of formate are oxidized per min per mole of selenium as selenocystine indicates a very efficient use of selenium or a very high turnover number.

The pathways involved in selenium utilization in bacteria, which could give some insight into the nature of the selenium requirement of FDH, have not been conclusively established. Clearly, selenite may be reduced in *E. coli* with production of elemental selenium (12, 25) and organic selenides in the form of seleno-amino acids (3, 33, 34). Painter (18) has suggested that elemental selenium may be produced by reaction of selenite with sulfhydryl groups. Selenite is known to be a substrate for the enzyme which reduces sulfite to sulfide in the pathway for incorporation of sulfur into amino acids (9); however, it is not certain that the seleno-amino acids are formed by this system. Selenate is not known to be reduced in *E. coli* (12). This may be a reflection of the instability of adenosine 5'-phosphoselenate (the product of the first enzyme in the sulfate reducing system) reported by Wilson and Bandurski (35). Knowing then that selenium may be present in the cell in a number of inorganic and organic oxidation states, it is interesting to speculate as to the nature of its function in FDH. If it is an integral part of the enzyme, selenium could have a catalytic role as selenocysteine (or selenocystine). It could also be present as non-heme iron selenide as proposed by Diplock et al. (5) for the acid-labile selenide of particulate proteins of rat liver. Selenide has been shown to replace the acid-labile sulfide of putidaredoxin with retention of biological activity (32).

Investigations are in progress in this laboratory to directly determine if FDH contains selenium and molybdenum and to define their role in the formation of this enzyme.

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