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Effects of Molybdate and Selenite on Formate and Nitrate Metabolism in *Escherichia coli*

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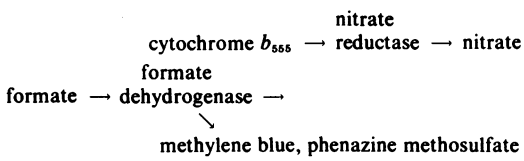
The effects of adding molybdate and selenite to a glucose-minimal salts medium on the formation of enzymes involved in the anaerobic metabolism of formate and nitrate in *Escherichia coli* have been studied. When cells were grown anaerobically in the presence of nitrate, molybdate stimulated the formation of nitrate reductase and a *b*-type cytochrome, resulting in cells that had the capacity for active nitrate reduction in the absence of formate dehydrogenase. Under the same conditions, selenite in addition to molybdate was required for forming the enzyme system which permits formate to serve as an effective electron donor for nitrate reduction. When cells were grown anaerobically on a glucose-minimal salts medium without nitrate, active hydrogen production from formate as well as formate dehydrogenase activity depended on the presence of both selenite and molybdate. The effects of these metals on the formation of formate dehydrogenase was blocked by chloramphenicol, suggesting that protein synthesis is required for the increases observed. It is proposed that the same formate dehydrogenase is involved in nitrate reduction, hydrogen production, and in aerobic formate oxidation.

Anaerobic electron transport in *Escherichia coli* and related organisms has received increasing attention in recent years because it represents a dispensable, integrated, membrane-associated system in which insight into the mechanisms of synthesis, assembly, and regulation might be gained by a biochemical-genetic approach. The biochemistry and genetics of the system responsible for nitrate reduction have received particular attention, partly because of the relative ease of obtaining mutants deficient in nitrate reduction (21, 24).

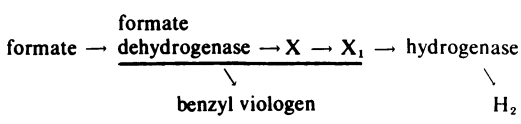
A pathway of electron transport has been proposed which reflects the observation that formate is the most effective electron donor for nitrate reduction (10, 23, 27, 28):



It has been shown that the level of activity of this system is regulated by the level of oxygen and nitrate in the grown medium (25, 28).

Another multienzyme system responsible for anaerobic hydrogen production is formate hydrogenlyase which catalyzes the reaction $HCOOH \rightarrow H_2 + CO_2$.

One proposed (8, 15) scheme for electron transport is



The classical requirements for the formation of this system are anaerobiosis, complex media, low *p*H, and the absence of nitrate (9, 16, 17).

A possible relationship between these two systems has been suggested by the finding of pleiotropic mutants of *E. coli* and related organisms which have lost not only all of the known components of the nitrate reductase system but also formate hydrogenlyase activity (14, 24, 26).

Pinsent reported in 1954 (22) that trace levels of molybdate and selenite are required for the formation of formate dehydrogenase in aerobically grown cells or nitrate-grown cells. However, with few exceptions (5), most workers have not added these trace components to defined anaerobic growth media and have reported, in general, much lower activities for the nitrate reductase system (28), the formate hydrogenlyase system (9, 18), or aerobic formate oxidation (12). We have found that both these anaerobic systems can

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be formed at high levels with a simple, defined medium that includes both molybdate and selenite.

MATERIALS AND METHODS

Culture methods. *E. coli* HfrH (*thi*$^-$) was used throughout and was obtained from D. Helinski. Two concentrated stock solutions contained some of the basal components of the culture medium. Solution 1 was composed of 229 g of $K_2HPO_4 \cdot 3H_2O$, 25 g of $(NH_4)_2SO_4$, and water to 1 liter. Solution 2 was composed of 2.5 g of $MgSO_4 \cdot 7H_2O$, 50.0 g of KH_2PO_4, 10.0 g of sodium citrate $\cdot 2H_2O$, 25 mg of ferrous ammonium sulfate, and water to 1 liter. What will be referred to hereafter as the basal anaerobic growth medium was prepared as follows. Autoclaved together, per 1 liter of final medium, were 40 ml of solution 1, 40 ml of solution 2, 50 ml of 2 M $KHCO_3$, 100 ml of 10% (w/v) KNO_3 (when noted), and an appropriate volume of water. To this sterile solution was added filter-sterilized thiamine hydrochloride to a final concentration of 5 μg/ml. In all experiments, the carbon source was D-glucose at a final concentration of 1% (w/v) added as a separately autoclaved 25% (w/v) solution.

Na_2SeO_3 and $Na_2MoO_4 \cdot 2H_2O$ were either autoclaved with the mineral salts or added filter-sterilized during the course of growth as later indicated.

Unless otherwise noted, anaerobic growth at 37 C was carried out in 1-liter glass reagent bottles fitted with a rubber stopper with appropriate ports for no. 14 gauge Teflon tubing for gassing and liquid sample removal. The cultures were continually gassed with 95% N_2-5% CO_2, 0.15 liter per min per vessel. To remove residual oxygen, the gas was treated with BASF catalyst R 3-11 according to the recommendations of the manufacturer (BASF Corp., Paramus, N.J.). The final, sterile culture medium was gassed for 1 hr or more before inoculation, at which point the *p*H was 8.1 to 8.2. A 1 to 4% (v/v) inoculum was employed which was grown anaerobically to mid-exponential growth phase in the medium described above, with or without KNO_3 as appropriate, and always in the absence of molybdate and selenite. Turbidity was measured with a Klett-Summerson colorimeter with a green filter; samples were diluted when necessary to always give a reading of less than 100; a reading of 1.0 corresponded to approximately 1.5 μg of protein per ml. Samples of culture medium used for estimation of total nitrite concentration were rapidly diluted with ice water followed by addition of the acidic nitrite reagent (25). Samples for subsequent enzyme analysis were harvested in the cold by centrifugation, washed twice with 50 mM sodium phosphate (*p*H 7.2), resuspended in this buffer to a concentration of 5 to 15 mg of protein per ml, and stored at −15 C.

Chemical estimations. Nitrite was estimated as previously indicated (25). Cell protein was estimated by the procedure of Lowry et al. (13) with bovine serum albumin as the standard after preincubation in 1 N NaOH for 30 min at 37 C.

Enzyme assay procedures. All assays were performed at 37 C and, with the exception of reduced nicotinamide adenine dinucleotide (NADH)-nitrate reductase, were carried out with frozen and thawed cell suspensions.

Nitrate reduction with formate as electron donor was carried out as previously described by measuring formate-induced nitrite production in an atmosphere of argon (24).

Formate hydrogenlyase activity was estimated by measuring H_2 evolution in a Warburg apparatus. The main compartment contained cells, 200 μmoles of sodium phosphate (*p*H 7.2), and water to 2.9 ml. CO_2 was trapped by 0.2 ml of 6 N KOH in the center well. After gassing with argon for 10 to 15 min, the stopcocks were closed and readings were taken for 10 min at which time either 0.1 ml of 0.6 M sodium formate or 0.1 ml of water was added from the side arms. Gas evolution was measured for 30 min, and the final value was corrected for a small endogenous gas evolution.

Nitrate reductase activity was measured by following the oxidation of reduced benzyl viologen by nitrate or chlorate spectrophotometrically. This measurement and those described below were carried out in an argon atmosphere in cuvettes that permitted anaerobic additions of small liquid volumes. A reaction mixture of 4 ml contained 0.4 mM benzyl viologen chloride, 0.1 mM disodium ethylenediaminetetraacetic acid (EDTA), and 49 mM sodium phosphate, *p*H 7.2. After the mixture was bubbled with argon for 2 min (100 ml/min, purified with BASF catalyst), reduced benzyl viologen was formed by the addition of 2 μliters of a freshly prepared solution of 0.25 M $Na_2S_2O_4$-0.52 M $NaHCO_3$. The absorbance of the solution was followed at 600 nm with a Gilford recording spectrophotometer. Enzyme (5 to 50 μliters) was added, and the endogenous rate was recorded for 30 to 60 sec. Oxidation of the reduced benzyl viologen was initiated by the addition of 15 μliters of 2.5 M $NaNO_3$ or 5 μliters of 5 M $NaClO_3$. The absorbance changes either before or after enzyme addition were quite small (<0.002 per min). The levels of nitrate and chlorate employed or 0.1 mM $NaNO_2$ did not cause any significant rate of reoxidation when added in the absence of enzyme. The initial rate of the reaction was observed to be directly proportional to the amount of enzyme added up to the highest levels of enzyme tested which gave a change of absorbance of 1.60 per min. A value of 7.4 mM^{-1} cm^{-1} obtained from a hydrosulfite titration was used for the extinction coefficient of the benzyl viologen mono cation, i.e., for a one-electron change. This compares well with values reported for methyl viologen (11, 29). These results (and those below) were calculated on the basis of a two-electron change.

Oxidation of NADH by nitrate was carried out anaerobically and followed at 340 nm. The reaction mixture (4 ml) containing 49 mM sodium phosphate (*p*H 7.2), 0.1 mM disodium EDTA, and 0.25 mM NADH was gassed for several minutes with argon. Enzyme (1 mg of protein) was added, and the endogenous rate was recorded for several minutes. The final reaction was started by the addition of 15 μliters of 2.5 M $NaNO_3$. For this assay only, just before assay, the frozen and thawed cells (5 mg of protein/ml) were sonically treated with five 10-sec pulses with the microtip of the Branson Sonifier at the no. 7 intensity setting.

The reaction mixture (4 ml) for formate-benzyl viologen reductase was composed of 49 mM sodium phosphate (*p*H 7.2), 0.25 mM sodium EDTA, and 1.0 mM

benzyl viologen chloride. This was gassed with argon for 2 to 3 min and 0.5 to 1.0 μliter of 0.25 M $Na_2S_2O_4$-0.52 M $NaHCO_3$ was then added followed by the appropriate amount of enzyme. The further reduction of benzyl viologen was initiated by the addition of 15 μliters of 4.8 M sodium formate. The reaction was monitored at 600 nm and calculated as noted above. It was found useful to add a small amount of sodium hydrosulfite before the enzyme addition. Otherwise, lags in reduction were observed whose duration was inversely proportional to the amount and activity of the enzyme added. These lags, presumably due to residual traces of oxygen, were virtually eliminated when the hydrosulfite was added. The initial rates observed were proportional to enzyme concentration. Only with enzyme preparations of very low specific activity (1/50 of the highest) did there seem to be a lag even in the presence of hydrosulfite; it should be noted that in these cases without hydrosulfite the lags observed were of 15 to 60 min in duration, quite impractical for routine spectrophotometric assays.

Formate dehydrogenase was also measured by dichlorophenolindophenol (DCPI) reduction mediated by phenazine methosulfate (PMS). The assay was similar to that previously described (24); the main difference was that it was carried out anaerobically. To the anaerobic cuvette was added 3.85 ml of a solution of 51 mM sodium phosphate (*p*H 7.2), 0.078 mM DCPI, and 0.1 ml of a freshly prepared solution of PMS (3 mg/ml). This mixture was gassed with argon for several minutes, and then the absorbance was followed at 600 nm. Enzyme was added and the endogenous rate of reduction was measured for 1 to 2 min. The reaction proper was initiated by the addition of 15 μliters of 4.8 M sodium formate. The endogenous rate was subtracted from the rate with substrate, and for the final calculations an extinction coefficient of 21,000 $M^{-1}\,cm^{-1}$ was used.

Absorption spectra of cells. Spectrophotometric measurements were made with a single-beam spectrophotometer in line with a PDP-81 computer (2). All spectra were of hydrosulfite-reduced cells (10 mg of protein per ml of 50 mM sodium phosphate, *p*H 7.2) at liquid nitrogen temperature with a path length of 0.63 cm. Fourth-derivative spectra were presented to enhance the resolution of spectral components in a complex spectrum. Maxima in the fourth-derivative spectra correspond to absorption maxima of the original bands, but the half-width of the fourth-derivative bands is considerably less than the half-width of the absorption bands. The methods for obtaining fourth-derivative spectra and the interpretation of the results have been discussed (3).

Source of chemicals. $K_2HPO_4 \cdot 3H_2O$, ferrous ammonium sulfate, and $Na_2MoO_4 \cdot 2H_2O$ were from Mallinkrodt Chemical Works; $MgSO_4 \cdot 7H_2O$, KH_2PO_4, and sodium citrate $\cdot 2H_2O$ were from J. T. Baker Chemical Co.; $(NH_4)_2SO_4$ (special enzyme grade) was from Mann Research Laboratories; Na_2SeO_3 was from Nutritional Biochemicals Co.; thiamine hydrochloride was from Calbiochem; D-glucose, anhydrous, was from Matheson, Coleman and Bell.

RESULTS

Formate was found to be an effective donor for the reduction of nitrate or phenazine methosulfate with anaerobically grown cells only when the simple medium was supplemented with nutrient broth (Table 1, Experiment 1). Similar findings have been noted by other workers who have measured the various enzymatic activities associated with formate and nitrate metabolism; however, some substantial activity was usually observed with simple media (5, 10, 16, 17, 24, 28).

In 1954 Pinsent (22) showed that, when various *coli-aerogenes* strains were grown aerobically or in standing culture with nitrate, on a simple medium whose components had been rigorously purified, the oxidation rate of formate could be increased from 15 to 150 nmoles per min per mg (dry weight) when both selenite and molybdate (10^{-8} to 10^{-7} M) were included in the growth medium. Formate oxidation was measured by following oxygen uptake manometrically. The level of formate-methylene blue reductase was also increased by selenite plus molybdate in the growth medium. Likewise, Fukuyama and Ordal (5) showed that formate dehydrogenase activity measured manometrically with methylene blue increased from 70 to 250 nmoles per min per mg (dry weight) when the iron-deficient aerobic growth medium was supplemented with selenium and molybdenum. These findings led us to examine the effects of these trace metal supplements on the levels of additional enzyme activities associated with formate and nitrate metabolism.

It can be seen (Table 1, experiment 2) that formate was an effective electron donor for nitrate reduction or phenazine methosulfate reduction when cells were grown on the simple defined medium supplemented with both molybdate and selenite at a level of 10^{-6} M. The presence of nitrate in the medium also greatly stimulated these activities. It has been shown (18, 25, 27) that reduced viologen dyes are effective electron donors for nitrate reduction by the *E. coli* nitrate reductase at all stages of purity. It has also been shown that chlorate can be reduced by the nitrate reductase of *E. coli* and related organisms (20). The assay employed in the present studies permits direct observation of reduced benzyl viologen oxidation by either nitrate or chlorate. It can be seen that a substantial activity (with either chlorate or nitrate) was observed without added metals, and a greater stimulation of both activities was observed with the sole addition of molybdate to the growth medium. These findings suggest that the basal medium employed is not totally deficient in molybdate, whereas for practical purposes it seems totally devoid of selenite. As expected from previous work (25, 28), nitrate was also required to yield the highest levels of nitrate (or chlorate) reductase activity. Of particular in-

terest is that high levels of nitrate reductase activity could be achieved in the absence of any formate dehydrogenase activity. Previous work in a number of laboratories (4, 10, 24, 28) had indicated that formate was the principal, if not the sole, electron donor for nitrate reduction in anaerobically grown *E. coli*. It was therefore of interest to ask whether cells growing in the absence of selenite (and thus without active formate dehydrogenase) used their high levels of nitrate reductase to reduce nitrate with a donor other than formate.

In the experiment shown in Fig. 1, cells growing exponentially on defined medium received additions of either selenite or molybdate or both. Nitrite concentration was measured for three to four cell doublings. In the bottom part of Fig. 1, the data are recalculated as specific activities: nanomoles of nitrite accumulated per minute per milligram of protein. It can be seen that with either molybdate alone or with molybdate plus selenite similar high specific activities were achieved. On the other hand, with no additions or with just selenite, the specific activity began to decline when the culture turbidity reached a Klett reading of 100 to 150. The findings are consistent with the notion already expressed above that the basal medium contains a low level of molybdate; that is, growth must proceed beyond a certain level for the molybdate to become limiting. Thus, it is concluded that cells growing in the absence of selenite with adequate molybdate carry on a quite substantial nitrate reduction. When selenite was added with molybdate, acid production was decreased as judged from the *p*H of the culture medium (Fig. 1, top). We interpret this as a reflection of formic acid oxidation, coupled to nitrate reduction.

The data in Table 1 were derived from cells harvested in early stationary phase. It was felt worthwhile to examine the various enzyme specific activities during exponential anaerobic growth in the presence of nitrate upon addition of selenite and molybdate. The results can be seen in Fig. 2. The upper portion of the figure shows that, although addition of selenite plus molybdate had no immediate effect on the growth rate, it did lead to a cessation of exponential growth at a somewhat lower cell density. Although not directly shown here, in all experiments such as this, significant growth inhibition began when the nitrite concentration reached 20 mM. The bottom portion of Fig. 2 was obtained by harvesting cells at the times indicated before and after trace metal addition. In the absence of added metals, the benzyl viologen-nitrate reductase specific activity decreased during exponential growth; again, this is consistent with exhaustion of limiting molybdate levels in the medium as growth

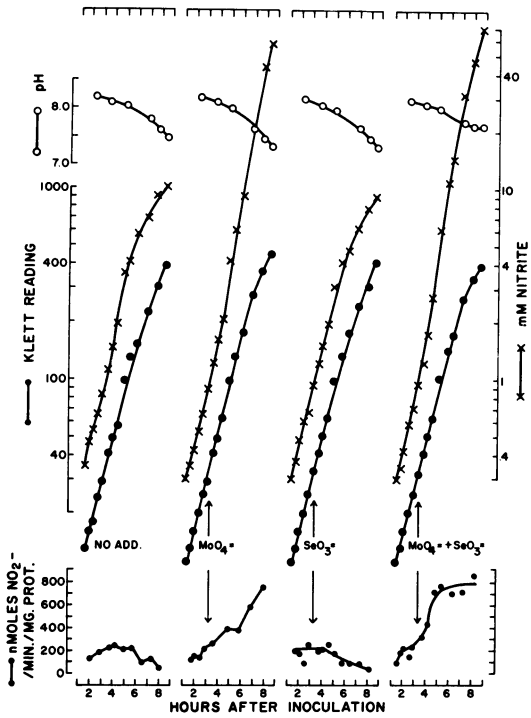

FIG. 1. *Effect of metal supplements during exponential growth on nitrite formation and pH of the medium. Four cultures were inoculated at zero time in the nitrate-containing basal anaerobic growth medium and cultured as indicated in Materials and Methods. At the times noted, selenite and molybdate were added to a final concentration of* 10^{-6} *M.*

proceeds. It can be seen that addition of the metals resulted in immediate increases in the specific activity of formate dehydrogenase, benzyl viologen-nitrate reductase, and formate-nitrate reductase during exponential growth. It should be noted that these measured specific activities were several-fold higher than the in vivo nitrite accumulation activities (Fig. 1). This in vivo activity is necessarily related to the overall rate of glucose metabolism during growth.

It has been shown that electron transport from formate to nitrate in *E. coli* involves one or more molecular forms of a *b*-type cytochrome (4, 10, 23) and that the level of this cytochrome *b* increases upon addition of nitrate to the anaerobic growth medium (4, 23, 28). It was of interest, therefore, to ask whether this cytochrome was also present in cells grown on defined medium and whether its presence was in any way regulated by the presence of trace metals in the medium. Figure 3 shows the absorption spectrum of hydrosulfite-reduced *E. coli* cells grown on defined medium. The spectra were taken at liquid N_2 temperatures. It can be seen that molybdate-grown cells have a very intense band at 555.5 nm,

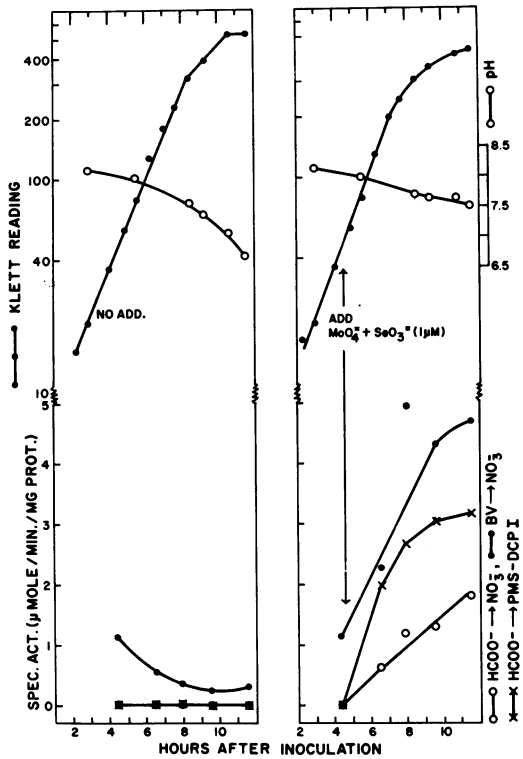

FIG. 2. *Effect of metal supplements during exponential anaerobic growth with nitrate on several enzyme activities. Experimental conditions as in Fig. 1 and Materials and Methods. BV, benzyl viologen.*

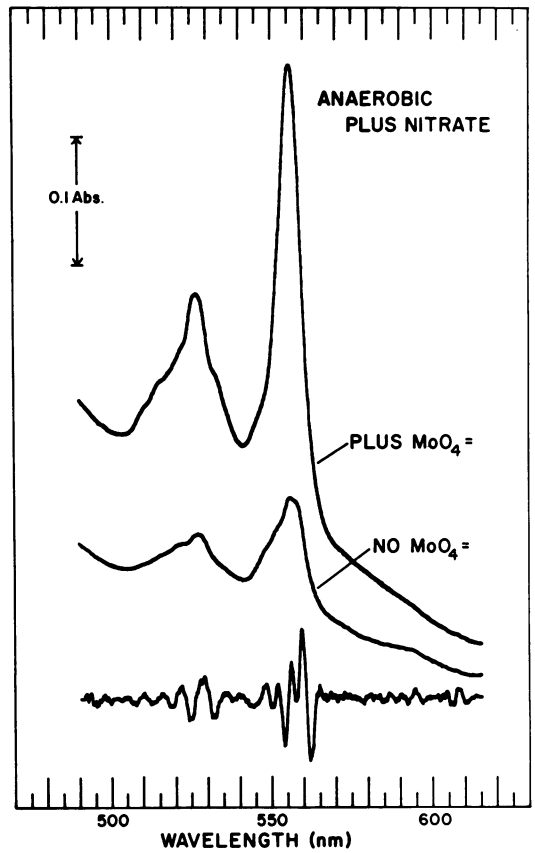

FIG. 3. *Effect of molybdate in the growth medium on the absorption spectra of cells grown anaerobically with nitrate. Details of the spectroscopy are given in Materials and Methods. The spectrum of cells grown with no metal supplement was identical in every way to the one shown above with selenite alone. Likewise the spectrum of cells grown with only molybdate is virtually identical to that shown above for selenite plus molybdate. The only difference was a slightly lower (10 to 20%) overall intensity. These are omitted in the interest of brevity.*

whereas cells from relatively molybdate-deficient medium (middle curve) show about 1/5 the intensity at this wavelength. The actual difference is probably much larger, since the spectrum of the molybdate-deficient cells is a complex mixture of several pigments. This can be more clearly seen in the fourth-derivative spectrum (bottom curve) of the molybdate-deficient cells which show three additional maxima at 559, 551.5, and 547 nm. These first two bands undoubtedly contribute significantly to the total absorbance at 555.5 nm. Thus, the absolute difference might approach 15-fold which is the approximate molybdate effect on the specific activity of the benzyl viologen-nitrate reductase activity (Table 1). It is clear then that when nitrate and selenite are present molybdate simultaneously regulates the levels of formate dehydrogenase, nitrate reductase, and cytochrome b_{555}.

Another important aspect of anaerobic formate metabolism in *E. coli* involves the formate hydrogenlyase system responsible for the conversion of formate to CO_2 plus H_2. Classically, this system is formed anaerobically in the absence of nitrate on complex media at an acid *p*H (8, 9). Although all of the individual components of this system have not been characterized in detail, the minimal components appear to be a formic dehydrogenase that can pass electrons to viologen acceptors and a hydrogenase immediately responsible for the evolution of H_2 (8). Workers in this field have distinguished the formate dehydrogenase of the formate hydrogenlyase system from the aerobic or nitrate-induced formic dehydrogenase based on electron acceptor specificity and solubility of the enzymes (8). In the present work, we have addressed ourselves only to the question of whether the activities of the formate hydrogenlyase system depend on the presence of trace metals in the growth medium. When cells were grown in standing culture in defined medium in

TABLE 1. *Effect of anaerobic growth medium supplements on the levels of enzyme activities related to nitrate reduction*[a]

Expt	Supplements to the basal anaerobic growth medium				Specific activity (nmoles per min per mg of protein) with electron donor/acceptor pairs				
	KNO_3 (+%)	Nutrient broth (0.4%)	Na_2MoO_4 (10^{-6} M)	Na_2SeO_3 (10^{-6} M)	Formate/ nitrate	Formate/ PMS-DCPI	NADH/ nitrate	Benzyl viologen/ nitrate	Benzyl viologen/ chlorate
1	+	−	−	−	<3	<1			
	+	+	−	−	420	960			
2	+	−	−	−	<4	<4	50	260	240
	+	−	+	−	<2	<1	130	1,900	3,450
	+	−	−	+	<2	110	40	270	210
	+	−	+	+	1,300	3,000	120	4,150	9,500
	−	−	+	+	29	210		35	500

[a] Composition of the basal medium, the routine anaerobic growth procedure, and the enzyme assays were those described in Materials and Methods. Cells were harvested 1 to 2 hr after exponential growth had ceased. Abbreviations: PMS, phenozine methosulfate; DCPI, dichlorophenolindophenol; NADH, reduced nicotinamide adenine dinucleotide.

the absence of trace metals, no activity could be found for hydrogen production from formate, formate-benzyl viologen reductase, or formate-PMS reductase. Addition of either nutrient broth or molybdate plus selenite resulted in substantial activity in all three assays (Table 2). Although not shown here, gas production measured with an inverted tube in the classical manner was done in each case and the results correlated perfectly with H_2 production from formate measured in the Warburg apparatus. A growth experiment was also carried out in a controlled atmosphere of 95% N_2-5% CO_2 (Table 2, experiment 2) which showed that, under these conditions, the formate-hydrogenlyase activity measured by H_2 evolution, formate-benzyl viologen reductase, and formate-PMS reductase activities all depend on the presence of both molybdate and selenite in the growth medium; further addition of KNO_3 strongly inhibited the benzyl viologen reductase but increased the PMS reductase activity. Nitrate is known (4) to repress hydrogenase and hydrogenlyase formation. Although leading to elevated formate dehydrogenase levels, nitrate somehow leads to the loss of the ability to couple this dehydrogenase to benzyl viologen reduction. We conclude that formate dehydrogenase activity both in the presence and absence of nitrate depends on both molybdate and selenite.

The cytochrome absorption spectrum of cells grown anaerobically in the absence of nitrate was measured at liquid N_2 temperature. Shown in

TABLE 2. *Effect of supplements to the anaerobic growth medium on the enzyme activities related to the formate hydrogenlyase system*[a]

Expt	Supplements to the basal medium					Specific activity with formate as electron donor (nmoles per min per mg of protein)		
	Nutrient broth (0.4%)	Na_2MoO_4 (10^{-6} M)	Na_2SeO_3 (10^{-6} M)	KNO_3 (1%)	Final *p*H of medium	Hydrogen production	Benzyl viologen reduction	PMS-DCPI reduction
1	−	−	−	−	5.2	0	<1	6
	+	−	−	−	5.3	70	5	103
	−	+	+	−	5.5	200	80	132
2	−	−	−	−	6.1	1	<3	<4
	−	+	−	−	6.2	1	<3	3
	−	−	+	−	6.2	5	<3	9
	−	+	+	−	6.4	45	150	130
	−	+	+	+	7.7		10	1900

[a] In experiment 1, cells were harvested after 13 hr of growth in standing culture (large test tubes) in the basal medium (minus $KHCO_3$) described in Materials and Methods. The initial *p*H was 7.1. In experiment 2, cells were grown anaerobically in an atmosphere of 95% N_2-5% CO_2 with the basal medium (*p*H 8.2) as described in Materials and Methods. The cells were harvested about 2 hr after exponential growth had stopped (Klett reading of 400 to 450). Abbreviations: PMS, phenazine methosulfate; DCPI, dichlorophenolindophenol.

Fig. 4 are the spectra from cells grown with selenite (bottom) and cells grown with selenite plus molybdate (top); the principal difference is that molybdate increases the amount of a *c*-type cytochrome with a 549.5-nm maximum. This absorption maximum corresponds to the low-potential cytochrome *c* previously studied (7). Two other maxima are evident in both types of cells at 555.5 nm and at 558.5 nm. A role for a low potential cytochrome in H_2 production has been suggested for *Desulfovibrio desulfuricans* and *E. coli* (8).

Mechanism of action of selenite and molybdate. Starting with cells grown on unsupplemented medium or supplemented with either molybdate or selenite, attempts were made in vitro to restore either nitrate reductase or formate dehydrogenase activity. We added sodium selenite or sodium molybdate, or both, at various concentrations before or during enzyme assay to both frozen and thawed cells and crude extracts prepared by sonic oscillation or by spheroplast lysis. All of these experiments were unsuccessful. That protein synthesis is required to show these metal effects can be seen from the data of Table 3. The addition of chloramphenicol to an actively growing culture along with both molybdate and selenite effectively inhibited the production of formic dehydrogenase activity. It can also be seen that formate could not replace selenite in inducing activity; in fact, it slightly reduced the activity evoked in the presence of molybdate and selenite. One logical possibility was that the metal effects were at the level of formate production, perhaps affecting pyruvate lyase. The absence of formate as an inducer could then have explained the low level of formate dehydrogenase. These experiments rule out this possibility.

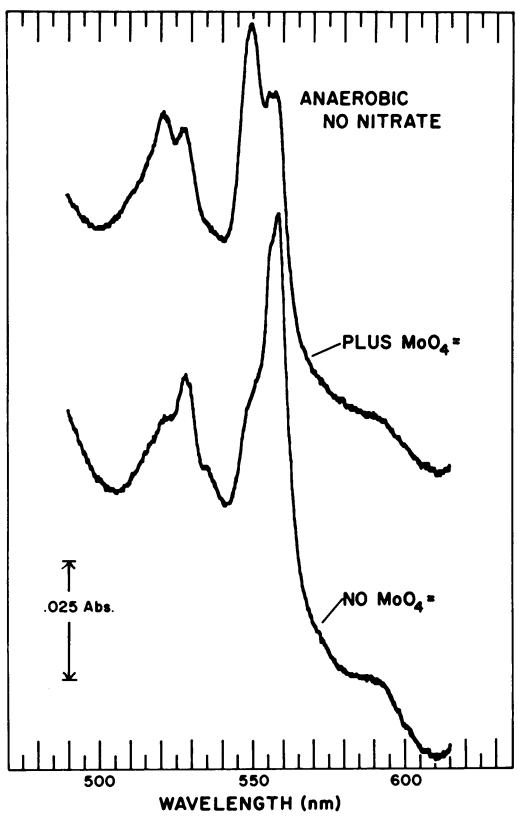

FIG. 4. *Absorption spectra of cells grown anaerobically without nitrate. Details of the spectroscopy are given in Materials and Methods. Spectra were also taken of cells with no supplement and with molybdate alone; these are not shown for the reason given in the legend to Fig. 3.*

DISCUSSION

These results show that a simple, defined medium is at hand in which *E. coli* can be grown anaerobically and have a high capacity for the multienzyme systems responsible for nitrate reduction and hydrogen evolution. No special precautions were taken in the preparation of the medium; off-the-shelf, reagent grade chemicals were used throughout. It was surprising, therefore, to encounter such large effects by the addition of trace levels of selenite and molybdate in view of the rigorous precautions taken by Pinsent (22) to obtain comparable effects. Part of the difference may reflect better overall quality of current commercial chemicals. It is also clear that the high growth densities achieved also played a role in making the metals, particularly molybdate, limiting. One batch of anhydrous K_2HPO_4 employed appeared to have enough molybdate so that a requirement could not be demonstrated. In all experiments to date, for all practical purposes, the requirement for selenite was absolute. Higher anaerobic growth yields (two to three times) were obtained starting at *p*H 8.2 than at *p*H 7.1. Whether this reflects a response to *p*H per se or to bicarbonate concentration remains to be decided by future experiments.

The mechanism by which the trace metals exert their effects is largely a matter of speculation. Although it is probable from the work of Taniguchi and Itagaki (27) that molybdenum is an integral part of the nitrate reductase molecule, it is not at all clear in what manner molybdate regulates the level of the cytochrome *b* of the system. Pinsent (22) showed that the aerobic cells and nitrate-grown cells require both molybdate and selenite for the formation of formate dehydrogenase. We have confirmed this finding for nitrate-grown cells and extended it to cells grown anaerobically in the absence of nitrate. This finding suggests that a single formate dehydro-

TABLE 3. *Factors regulating formate dehydrogenase activity*[a]

Additions to basal anaerobic medium at zero time	KR and FDH specific activity (μmoles per min per mg of protein)					
	Zero time		100 Min		190 Min	
	KR	FDH	KR	FDH	KR	FDH
Molybdate	68	<0.02	195	<0.01	400	<0.01
Molybdate, formate	68	<0.02	190	<0.01	340	<0.01
Molybdate, selenite	68	<0.02	200	1.78	380	2.15
Molybdate, formate, selenite	68	<0.02	180	1.35	280	1.51
Molybdate, selenite, chloramphenicol	68	<0.02	72	0.01	72	0.02

[a] Five cultures were started on the basal anaerobic growth medium containing 1% KNO_3 according to the procedures described in Materials and Methods. At a Klett reading (KR) of 68, the various "zero time" additions were made to the following final concentrations: Na_2MoO_4, 1 μM; Na_2SeO_3, 1 μM; sodium formate, 47 mM; chloroamphenicol, 19 μg/ml. Samples for enzyme assay were obtained just before zero time and at 100 and 190 min later. Formate dehydrogenase (FDH) was measured with the dichlorophenolindophenol procedure.

genase is operative under all of the growth conditions mentioned and that selenite and molybdate regulate its formation either by virtue of being integral components of the active enzyme or in some other more indirect fashion, e.g., as a component of a regulatory macromolecule. Workers in this field (8) usually distinguish two formate dehydrogenases on the basis of whether benzyl viologen or methylene blue can act as electron acceptor or whether the activity is associated with the membrane. Peck and Gest (6, 15), who first recognized that hydrogenlyase activity is correlated with reduction of viologen dyes by formate, clearly recognized the possibility that a single protein component might be responsible for formate "activation" and that additional components are formed under anaerobic conditions that could link this dehydrogenase to viologen reduction. From the standpoint of simplicity, we would favor this latter view.

It is clear from the present work that formate need not be considered as an obligatory donor for nitrate reduction since selenite-deficient cells carry on a significant nitrate reduction in the absence of formate dehydrogenase. Data in Table 1 show that at least a part of the nitrate reduction could be at the expense of NADH oxidation. The highest rates of NADH-nitrate reductase activity obtained in crude extracts were, however, still 5- to 10-fold lower than the in vivo nitrite accumulation rates. The in vitro experiments on NADH-nitrate reductase were possibly not carried out under optimal conditions; on the other hand, we cannot exclude the possibility of other significant donors. An analysis of the products of fermentation might be revealing in this regard.

Since under appropriate conditions molybdate alone can regulate the level of both the nitrate reductase and hydrogenlyase systems, we would like to direct attention to the possibility that a mutation affecting some step in the processing of molybdate could give rise to a pleiotropic phenotype affecting both systems. Among many possibilities, this step could involve molybdate transport into the cell, oxido-reduction to the appropriate valence form, and finally incorporation of the element into its final form. This raises the possibility of finding distinct mutants that may map differently but have the same pleiotropic phenotype. Similarly, mutants affecting selenium processing might be encountered which would be expected to have an effect more restricted to formate dehydrogenase and formate hydrogenlyase. An alternative explanation put forward by Azoulay et al. (1), that a defect of a membrane assembly factor explains the pleiotropic effects, is by no means excluded by any of the data presented here.

We hope that the availability of a defined growth medium will aid in future work aimed at unravelling the many intriguing unsolved biochemical and genetic aspects of this problem.

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