THE INHIBITION BY NITRATE OF ENZYME FORMATION DURING GROWTH OF ESCHERICHIA COLI

DANIEL BILLEN[1]

Department of Bacteriology, University of Tennessee, Knoxville, Tennessee

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During an investigation of the effects of nutrients on formic hydrogenlyase activity in *Escherichia coli*, it was noted that the harvested cells possessed no measurable formic hydrogenlyase activity when grown in a basal medium composed of inorganic salts and glucose and containing NH_4NO_3 as the sole source of nitrogen. This was in agreement with the data reported in a previous communication (Billen and Lichstein, 1951). Addition of tryptone to the basal medium containing the NH_4NO_3 did not cause an expected increase in formic hydrogenlyase activity; however, when $(NH_4)_2HPO_4$ was substituted for NH_4NO_3 in the tryptone containing basal medium, the harvested cells contained formic hydrogenlyase activity. Since ammonium ions were present in both media and the phosphate concentrations were approximately the same, a nitrate inhibition of formic hydrogenlyase formation was suggested.

A review of the literature revealed several other observations of this nature. Pakes and Jollyman (1901) observed that during growth of *E. coli* in medium containing nitrate and formate no hydrogen was evolved, whereas gas formation was noted from cultures grown in the absence of nitrate. These authors assumed that this difference was due to the utilization of the hydrogen in the reduction of nitrate when present. Yudkin (1932) used resting cells to study hydrogen production from formate and from glucose and observed that the addition of nitrate to the broth medium in which the cells were grown inhibited the "production of the enzymes" responsible for the hydrogen evolution. Lee and Wilson (1943) noted that the presence of ammonium or nitrate ions in the culture medium caused a reduced hydrogenase activity in *Azotobacter* and the reduction paralleled a decrease in nitrogen fixation activity.

It has been observed (Billen and Lichstein, 1951) that formation of formic hydrogenlyase and hydrogenase requires the presence of certain amino acids during cell multiplication. With this information in mind, the present study was undertaken with the hope of elucidating the mechanism of nitrate inhibition of formic hydrogenlyase, hydrogenase, and formic dehydrogenase formation.

METHODS

The bacterial culture used was *Escherichia coli*, "Texas" strain. The methods of cultivation, harvesting, and washing of the organism and the assay of enzyme activities were the same as those previously described (Billen and Lichstein, 1951).

[1] Present address: Biology Division, Oak Ridge National Laboratories, Oak Ridge, Tennessee.

EXPERIMENTAL RESULTS

It was found that hydrogenase and formic dehydrogenase activity as well as formic hydrogenlyase activity were totally inhibited in the cells harvested from a basal medium[2] containing vitamin-free acid-hydrolyzed casein and varying concentrations of nitrate. All three enzymes vary in sensitivity to the inhibitor (table 1), formic hydrogenlyase being the most sensitive and, at lower concentrations of nitrate, formic dehydrogenase being the least sensitive. Not only did formic dehydrogenase prove to be the more resistant system, but low concentrations of nitrate stimulated formic dehydrogenase activity. A study of gas production during growth revealed that neither carbon dioxide nor hydrogen was produced in 24 hours from the cultures grown on the nitrate containing medium. Both gases were given off by the non-nitrate grown cells during the same period.

TABLE 1

The inhibition by nitrate of enzyme formation in Escherichia coli

EXPERIMENT	NH_4NO_3 ADDED TO MEDIUM*	PER CENT INHIBITION†		
		Hydrogenase	Formic dehydrogenase	Formic hydrogenlyase
	mg			
1	10	74	0‡	100
	50	100	100	100
	100	100	100	100
2	5	10	0‡	84
	10	65	0‡	100
	20	73	0‡	100

* Basal medium (100 ml) plus 10 ml of 10 per cent casein hydrolyzate.

† Based on a control without added nitrate.

‡ Stimulation noted.

A study of the effect of increasing concentrations of casein hydrolyzate was undertaken to determine whether the inhibition by nitrate was of a competitive nature with respect to amino acids required for enzyme formation. It was found that increasing concentrations of casein hydrolyzate overcome to varying degrees the nitrate inhibition effect (table 2). The enzyme systems appeared to exhibit different quantitative responses to the antagonizing properties of the casein hydrolyzate since formic dehydrogenase inhibition was most easily overcome while that of formic hydrogenlyase was most resistant to reversal.

Glutamic acid alone stimulates both hydrogenase and formic hydrogenlyase formation (Billen and Lichstein, 1951), hence an experiment was designed to determine whether this single amino acid could compete with the inhibitor.

[2] The basal medium had the following composition: 0.1 per cent KH_2PO_4, 0.07 per cent $MgSO_4 \cdot 7H_2O$, 0.1 per cent NaCl, 0.4 per cent $(NH_4)_2HPO_4$, 0.05 per cent citric acid neutralized with 10 per cent NaOH, 0.00005 per cent $FeCl_3$, and 1 per cent glucose added aseptically. The medium was adjusted to pH 6 to 7 before sterilization.

A 0.5 per cent concentration of glutamate gave no reversal of either hydrogenase or formic hydrogenlyase inhibition, whereas 1 per cent glutamate restored 10 per cent of the hydrogenase activity but none of the formic hydrogenlyase. It would appear that in this case glutamate alone is not as effective as casein hydrolyzate in reversing the nitrate inhibition effect.

It has been shown that hydrogenase formation takes place early in the growth cycle of *E. coli* (Billen and Lichstein, 1951). To determine whether the nitrate was exerting its effect during or after the period of greatest enzyme synthesis,

TABLE 2

The effect of increasing concentrations of casein hydrolyzate on nitrate inhibition of enzyme formation in Escherichia coli

CASEIN HYDROLYZATE ADDED TO MEDIUM*	PER CENT INHIBITION†		
	Hydrogenase	Formic dehydrogenase	Formic hydrogenlyase
ml			
10	95	100	100
30	75	0	95
40	26	0	38

* Basal medium (100 ml) plus 100 mg NH_4NO_3.

† Based on activity obtained from cells grown on basal medium (100 ml) plus 10 ml of casein hydrolyzate.

TABLE 3

The relationship between time of addition of the nitrate and inhibition of enzyme formation

AGE OF CULTURE AT TIME OF NITRATE ADDITION*	PER CENT INHIBITION†		
	Hydrogenase	Formic dehydrogenase	Formic hydrogenlyase
hr			
0	100	100	100
5	93	100	100
16	51	88	100

* 100 mg NH_4NO_3 added to cells growing in 100 ml of basal medium plus 10 ml of casein hydrolyzate.

† Based on activity obtained from cells grown in above medium to which no nitrate had been added.

nitrate was added to growing cultures during several phases of its growth cycle. The data obtained suggest that the nitrate acts by preventing the formation of biologically active hydrogenase. If the nitrate is added when the cells are 12 or more hours old or at a time when the period of greatest enzyme synthesis has passed, it no longer is as effective in its hydrogenase inhibition properties (table 3). A possible reason why inhibition is still evident when the nitrate is added after the period of most active enzyme synthesis may be that at this phase the cells are synthesizing hydrogenase to replace that which is being inactivated by normal means, and thus if nitrate is added, they are prevented from producing

the active enzyme molecules for replacement purposes. With the same reasoning in mind one may suggest that the replacement of formic hydrogenlyase and formic dehydrogenase goes on at an even more rapid rate, and thus the nitrate inhibition of these two enzymes is more pronounced during the later stages of the growth cycle.

DISCUSSION

Before any consideration of mechanisms of inhibition of enzyme formation by nitrate, it should be suggested that the inhibition may not be due to the nitrate *per se* but possibly to one of its reduction products such as nitrite or hydroxylamine. For discussion purposes only nitrate will be referred to.

One mode of action may be that the nitrate changes the direction of utilization of the amino acids by influencing a regulatory synthesis control mechanism in a similar manner to that which Spiegelman and Dunn (1947) have described as "competitive interaction". A recent paper by Wainwright (1950) lends some support to such a possibility since he has reported that the presence of casein hydrolyzate stimulated the formation of the adaptive enzyme, nitratase. This is of importance if it is noted that the concentration of nitrate (0.001 M) reported by Wainwright to cause maximal nitratase formation in *E. coli* is approximately equal to that concentration causing almost complete inhibition of formic hydrogenlyase and hydrogenase formation during growth (table 1). This could also explain why nitrate inhibition would be antagonized by increasing concentrations of amino acids since the competition for available amino acids would thus be reduced. It is possible, therefore, that there exists a protein synthesis control mechanism in which nitratase formation has precedence over formic hydrogenlyase, hydrogenase, and formic dehydrogenase in the utilization of available amino acids.

It is also possible that the nitrate selectively inhibits the formation of formic hydrogenlyase, hydrogenase, and formic dehydrogenase by directly interfering in the formation of a biologically active protein molecule or enzyme. Since little is known of the mode of enzyme synthesis and action, one can only speculate about such a possibility.

SUMMARY

The presence of nitrate in the culture medium inhibits the formation of hydrogenase, formic hydrogenlyase, and formic dehydrogenase in proliferating *Escherichia coli*. The time of addition of the nitrate to the growing cultures will influence its effectiveness as an inhibitor. Increasing concentrations of casein hydrolyzate antagonize the inhibition effect of the nitrate.

The possibility that nitrate acts by stimulation of nitratase formation thus causing the utilization of amino acids otherwise available for formic hydrogenlyase, hydrogenase, and formic dehydrogenase formation is discussed.

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