REQUIREMENTS FOR FORMIC HYDROGENLYASE ADAPTATION IN NONPROLIFERATING SUSPENSIONS OF ESCHERICHIA COLI[1]

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The adaptive character of formic hydrogenlyase, which catalyzes the reaction:

$$HCOOH \rightleftharpoons H_2 + CO_2$$

was first demonstrated with *Escherichia coli* by Stephenson and Stickland (1932, 1933). They were able to obtain adaptation without discernible cell division by incubating cell suspensions anaerobically with broth and formate. These experiments pointed to nutritional requirements for adaptation under conditions apparently uncomplicated by cell growth. We have determined the specific factors essential for this adaptation.

METHODS

Stock cultures of the test organism, *E. coli*, strain S, were maintained on 1 per cent yeast extract agar slants. To obtain cell suspensions, the total growth from a 24 hour slant was transferred to 100 ml of 1 per cent yeast extract broth in a 500 ml Erlenmeyer flask and incubated with rapid shaking for 8 hours at 28 C. Then a 1 per cent serial transfer was made into the same medium, and the culture was incubated under the same conditions as the first liquid culture. The cells were harvested after 12 hours, centrifuged, washed in distilled water, recentrifuged, and resuspended in M/10 phosphate buffer at pH 7.0 to give a final concentration of 1.5 mg of cells per ml on a dry weight basis. This is equivalent to a reading of 350 units on a Klett-Summerson photoelectric colorimeter using a 640 mμ filter. Since cells prepared in this manner could be stored in the refrigerator for a week without appreciable alteration, the cell suspensions were used either when freshly prepared or after storage for as long as 5 days.

Formic hydrogenlyase activity was measured in terms of H_2 evolution from formate under an atmosphere of N_2 in the Barcroft-Warburg apparatus at 28 C. In the standard procedure, each vessel received 2.0 ml of the unadapted cell suspension and 0.1 ml of M/10 Na formate, and also supplements of 0.1 ml of M/10 glucose and 0.2 ml of 10 per cent of vitamin free acid hydrolyzed casein in the side arm. Variations in the supplements and procedure are described in the text and tables. Formic dehydrogenase activity was measured in terms of O_2 uptake by the same standardized quantity of cell suspension with 0.2 ml of M/10 Na formate in air. Hydrogenase activity was measured in terms of H_2 uptake in an H_2 atmosphere with methylene blue as the hydrogen acceptor. To

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0.5 ml of the standardized cell suspension and 1.5 ml of the buffer in the main compartment, 1.0 ml of M/500 methylene blue was added from the side arm. All determinations were performed with 0.2 ml of 20 per cent KOH in the center well. Rates or Q values are expressed as microliters of gas evolved or taken up per hour per mg of cells, on a dry weight basis.

Two criteria can be used for comparing the effectiveness of the various supplements in inducing adaptation: the length of the lag period required for the development of maximum hydrogenlyase activity, and the magnitude of the latter. In our experiments, in general, higher maximum activities were accompanied by shorter lag periods. However, since the maximum activity was more easily determined experimentally and varied more markedly than the lag, it was used as the criterion.

In all of the synthetic amino acid mixtures, only L-isomers were used with the exception of DL-alanine, isoleucine, methionine, norleucine, phenylalanine, serine, threonine, and valine. All concentrations mentioned for the latter amino acids refer only to the amounts of the L-isomer.

RESULTS

The cell suspensions prepared as described were devoid of hydrogenlyase activity, owing to the aerobic conditions of growth and to the absence of added carbohydrate. Attempts to induce hydrogenlyase adaptation in them with any one of a large assortment of peptic or tryptic digests of various plant or animal materials such as lactalbumin, gelatin, muscle protein, and soya meal were unsuccessful. Upon the further addition of glucose, however, in all cases the evolution of hydrogen began within 30 minutes and gradually accelerated to reach a steady vigorous rate within 1 to 2 hours.

The results of a typical experiment in which the best defined supplements, vitamin free hydrolyzed casein and glucose, were used are presented in figure 1. With glucose alone, H_2 evolution was barely detectable, and with hydrolyzed casein alone activity did not develop.

Furthermore, mannitol and pyruvic acid could be satisfactorily substituted for glucose, but ammonia could not replace hydrolyzed casein. Thus, it appears that *E. coli* needs both energy and amino acids for formic hydrogenlyase adaptation. This behavior is analogous to that of yeasts (Spiegelman, 1951) which require both nitrogen and energy for the formation of adaptive enzymes for several sugars.

The effect of pH on the adaptive process was determined by allowing the cells to adapt in M/10 phosphate buffer at different pH values when placed in deep layers in stoppered tubes for 1½ hours. The cells were then centrifuged, resuspended in M/10 phosphate buffer at pH 7.0, and their activities determined with formate alone. There is some loss of activity resulting from washing of the cells. The values are plotted in figure 2 and indicate that maximum adaptation occurred at pH 6 to 7.

The affinity for glucose is shown in figure 3. Essentially maximum adaptation was obtained with a final concentration of M/120 glucose. The corresponding

affinity for hydrolyzed casein, as shown in figure 4, indicates that the standard concentration is close to optimum.

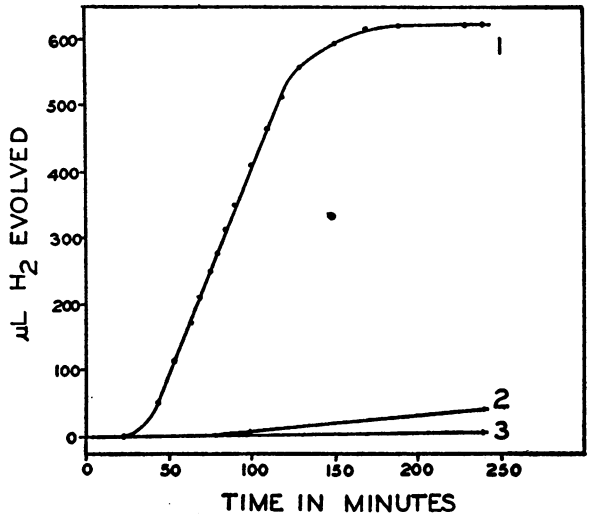

Figure 1. Adaptation of hydrogenlyase in *Escherichia coli* with exogenous nitrogen and energy. 1 = glucose plus hydrolyzed casein, 2 = glucose, 3 = hydrolyzed casein.

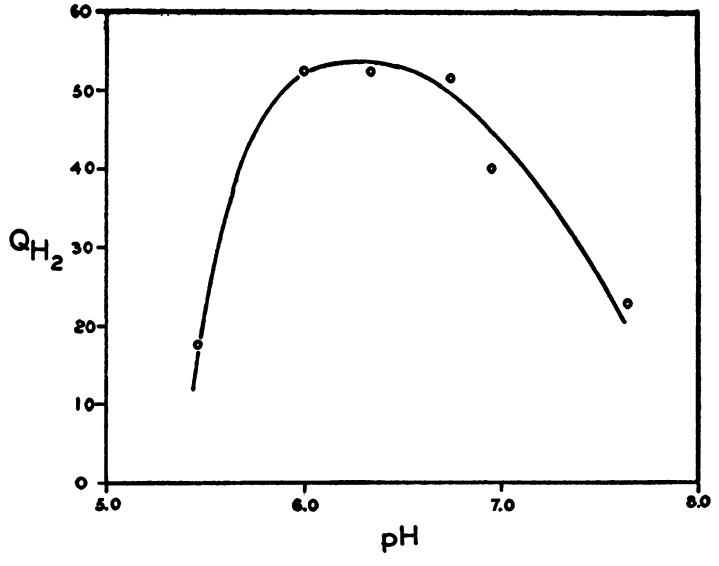

Figure 2. Effect of pH on the adaptation of formic hydrogenlyase.

During adaptation, there was no appreciable change in cell numbers as determined by plate counts, or in turbidity, which indicates that adaptation takes place without the occurrence of growth.

Amino acid requirements for hydrogenlyase adaptation. An amino acid mixture which approximated the composition of hydrolyzed casein (Sahyun, 1948) was as effective as the natural product as shown in table 1.

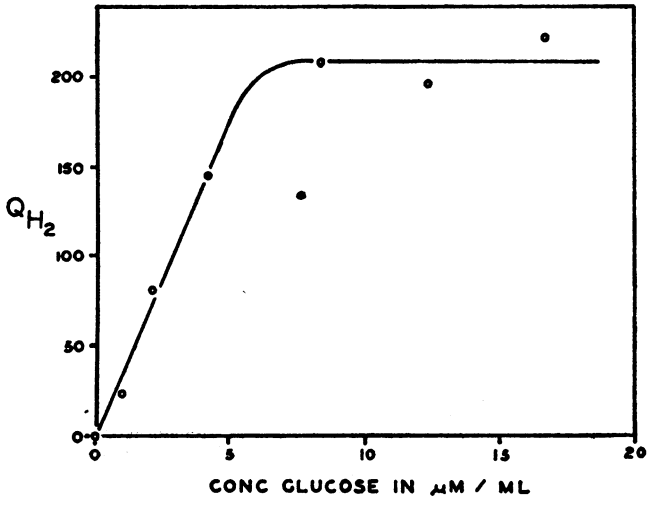

Figure 3. Affinity curve of glucose for formic hydrogenlyase adaptation. The filled in circle indicates activity obtained under standard conditions (see text).

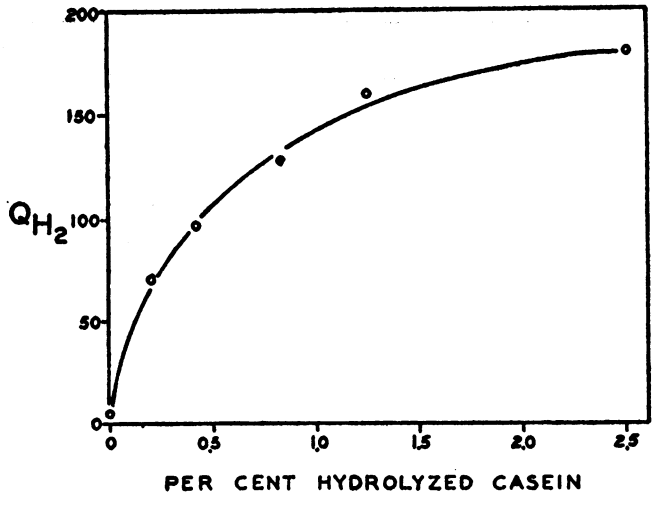

Figure 4. Affinity curve of hydrolyzed casein for formic hydrogenlyase adaptation. The filled in circle indicates activity obtained under standard conditions (see text).

Since digests of lactalbumin, gelatin, and casein were highly effective supplements, we tested a mixture of the 12 amino acids which occur in appreciable amounts in all of them, i.e., alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, phenylalanine, proline, and serine

in the amounts found in 1.66 per cent gelatin (Sahyun, 1948). This mixture possessed one-third the activity of the hydrolyzed casein standard. The omission of glycine, phenylalanine, proline, and serine had no effect on the activity. The further omission, individually, of each of the remaining amino acids did not alter activity by more than 10 per cent with the exception that omission of arginine, aspartic acid, or glutamic acid reduced activity to less than 10 per cent of the hydrolyzed casein standard. Therefore, these three amino acids are essential. Arginine, aspartic acid, or glutamic acid, when used individually and in the same amounts as in the hydrolyzed casein standard, supported very

TABLE 1

Adaptive activities of various amino acid substitutes for hydrolyzed casein

AMINO ACIDS (IN THE AMOUNTS FOUND IN 0.83 PER CENT HYDROLYZED CASEIN)	RELATIVE ADAPTIVE ACTIVITY
Vitamin-free hydrolyzed casein (natural)	100*
Hydrolyzed casein (synthetic)	100
Arginine, aspartic acid, or glutamic acid	6 to 10
Arginine, aspartic acid, and glutamic acid	62
Arginine, aspartic acid, cystine, glutamic acid, glycine, serine, and threonine	83

* 100 = Q_{H_2} 130.

TABLE 2

Effect of single amino acids on a mixture of arginine, aspartic acid, and glutamic acid on formic hydrogenlyase adaptation by Escherichia coli

STIMULATORY		INHIBITORY		INACTIVE	
Cystine	+26*	Cysteine	−40	Alanine	+5
Glycine	+19	Histidine	−15	Hydroxyproline	+1
Serine	+19	Isoleucine	−26	Lysine	−6
Threonine	+18	Leucine	−47	Ornithine	−4
		Methionine	−29	Proline	+3
		Phenylalanine	−33	Tryptophan	0
				Tyrosine	+3
				Valine	−5
				NH_4Cl	−1

* All amino acids were present in M/80 concentrations. Activity calculated as the per cent increase or decrease over the final Q_{H_2} of the 3 amino acid mixture alone.

little hydrogenlyase formation, but a combination of the three amino acids was highly active (table 1).

The effect of additional amino acids was investigated by adding them singly to a mixture of the three essential amino acids, the latter being present in maximally active concentrations. It appears (table 2) that cystine, glycine, serine, and threonine are stimulatory, 6 other amino acids are inhibitory, and the remainder, as well as ammonia, are inactive. A mixture of the 7 essential and stimulatory amino acids in the same amounts as found in the hydrolyzed casein standard can almost replace the complete product (table 1). If individual amino

acids are omitted singly from the 7 amino acid mixture at the same or at higher concentrations, there is always a loss in activity, indicating that the amino acids cannot replace each other.

An attempt was made to determine whether it was necessary for the amino acid and glucose supplements to be present simultaneously for adaptation. Formate and glucose may be added to the cells for as much as an hour before the addition of the hydrolyzed casein without any difference resulting in final hydrogenlyase activity, provided sufficient glucose is added so it is not depleted too much by fermentation prior to the addition of hydrolyzed casein. This pretreatment with the energy source and the substrate, however, shortens the lag period of adaptation within certain limits. A pretreatment of 10 minutes diminishes the lag by 10 minutes, but longer pretreatment periods have no additional effect. It appears that the nitrogenous resources of the cells are ample for the first 10 minutes of the adaptation process, but after that, external nitrogen must be supplied for further adaptation.

Conversely, if the addition of the glucose is delayed 2 hours after the addition of hydrolyzed casein and formate, the resulting hydrogenlyase activity is only very slight. This suggests that perhaps critical amino acids are decomposed by deamination or decarboxylation during the preincubation period. This possibility was tested by incubating amino acids singly with the cell suspensions plus glucose and formate for 2 hours anaerobically. Only aspartic acid and, to a lesser extent, serine were decomposed as evidenced by an increase in free ammonia. Furthermore, only glutamic decarboxylase of the amino acid decarboxylases was detectable and only in insignificant amounts when determined according to the method of Gale (1940).

It is possible that the deamination of the essential aspartate is responsible for the inactivation of the hydrolyzed casein during preincubation. If again the addition of the glucose is delayed for 2 hours, but supplemented with aspartic acid, the activity of the hydrolyzed casein is restored completely. In contrast, the other essential and stimulatory amino acids, either singly or in combination, have no restorative effect. This emphasizes the essentiality of aspartic acid and suggests that aspartic acid is used by the cell without previous alteration.

Neither the essential nor the stimulatory amino acids, nor cysteine, one of the most inhibitory amino acids, has any demonstrable effect on the hydrogenlyase activity of preadapted cells. Therefore, their action must be confined solely to the adaptive process.

The need for amino acids and an energy source for adaptation was established also for *E. coli*, strains Crookes, B, and mutabile. *E. coli*, strain K 12, on the other hand, develops only slight activity when both glucose and hydrolyzed casein are supplied.

The total yield of acid and the length of time required for the dissimilation of 3.33 μM of glucose are the same for adapted and unadapted cells, as determined manometrically by CO_2 liberation from bicarbonate buffer at pH 7.0 under an atmosphere of 5 per cent CO_2 and 95 per cent N_2. Therefore, there is no significant increase of glucozymase. Also, no acquisition of amino acid de-

carboxylase activity could be detected either under the standard conditions or at pH 5 which would be more favorable for amino acid decarboxylase formation (Gale, 1940). Furthermore, the formic dehydrogenase activity remains unaltered during adaptation. However, the Q_{H_2} of hydrogenase, which is initially 84 in the unadapted cells, increases to 370 during the adaptation. The effect of the supplements, therefore, is primarily on hydrogenlyase adaptation.

Hydrogenlyase adaptation is sensitive to assimilatory poisons. Thus, the metal-trapping agents α,α-dipyridyl and 8-hydroxyquinoline in M/1,200 concentration inhibit adaptation completely but have no effect on hydrogenlyase activity of adapted cells. Likewise, M/1,200 2,4-dinitrophenol inhibits adaptation almost completely but also inhibits the activity of preadapted cells by 74 per cent.

If the adaptation is permitted to take place in air instead of N_2, hydrogenlyase formation is completely prevented. However, previous aeration of the unadapted cells even for several hours does not alter their subsequent adaptive behavior under N_2, and hydrogenlyase once formed is not affected by aeration for 1 hour. Therefore, although adaptation is prevented by O_2, neither the enzyme nor the enzyme forming sytsem is destroyed by it.

The action of $NaNO_3$ or $NaNO_2$ on hydrogenlyase adaptation and activity is more complex than that of O_2. The cell is immediately permeable to all concentrations of NO_3^- and NO_2^- mentioned later, since the production of NO_2^- from NO_3^- by the action of nitrate reductase and the loss of NO_2^- by the action of nitrite reductase begin immediately on addition of these substrates and proceed linearly as determined with the Griess Ilosvay reagent at short time intervals. Eight hundred and thirty $\times 10^{-6}$ M NO_3^- or higher concentrations do not appear to inhibit completely the activity of preformed hydrogenlyase immediately, but only after a lag period of approximately 8 minutes. By the time inhibition is complete, 360×10^{-6} M NO_2^- has been produced. $NaNO_2$ (83×10^{-6} M) also inhibits adaptation completely when nitrite reductase is negligible. This inhibition also has a lag period of 10 to 15 minutes which can be abolished gradually by using progressively higher concentrations of NO_2^-. At concentrations below 83×10^{-6} M, NO_2^- inhibition is slight and rapidly reversed by the disappearance of NO_2^- due to the action of nitrite reductase. At such concentrations, NO_3^- parallels the action of NO_2^-, except for a slightly longer lag. Therefore, the formation of NO_2^- as an intermediate in NO_3^- reduction could explain the inhibition observed with NO_3^-. The role of NO_3^- *per se* is obscure since it is impossible to avoid the concomitant appearance of the interfering NO_2^-. Nitrate and NO_2^- do not completely inhibit formic hydrogenlyase either by exhausting the substrate or by providing a competitive oxidative pathway for formate dissimilation. Thus, the low concentrations of NO_3^- and NO_2^- used can oxidize only small fractions, as low as 3 per cent, of the total formate. The respective rates of formate consumption by nitrate reductase and nitrite reductase are 48 per cent and 5 per cent as great, respectively, as that of formic hydrogenlyase. Furthermore, in cells with lower rates of nitrite reduction the inhibition is more prolonged, presumably because the inhibitor is removed more slowly. When NO_2^-

is eventually exhausted, the more rapid, normal rate of formate consumption is resumed by the reactivated formic hydrogenlyase.

The nitrite reductase activity is immediate and is saturated at the lowest inhibitory concentration of NO_2^- tested, 40×10^{-6} M. Since higher concentrations than this, at least 830×10^{-6} M NO_2^-, are required to effect immediate and complete inhibition of H_2 formation, the excess NO_2^- could not be stimulating the formation of inhibitory intermediates, and hence the inhibition must be attributed directly to NO_2^-.

In unadapted cells, 830×10^{-6} M NO_2^- has no inhibitory effect on formic dehydrogenase activity when the latter is determined manometrically by CO_2 production from formate with methylene blue as hydrogen acceptor under N_2. The same concentration of NO_2^- has a negligible inhibitory effect on hydrogenase. However, since hydrogenlyase under these conditions is completely and immediately inhibited in the adapted cell, it appears that NO_2^- in low concentrations selectively inhibits formic hydrogenlyase.

$NaNO_3$ or $NaNO_2$ in quantities sufficiently large so it is not exhausted during adaptation almost completely inhibits the formation of hydrogenlyase. The activity of the latter was measured after removing the nitrite by centrifuging and washing the cells. Thus, nitrate and nitrite interfere with both adaptation and activity of formic hydrogenlyase.

DISCUSSION

Our results indicate that both a source of energy and nitrogen are necessary for the adaptive formation of formic hydrogenlyase. Since Stephenson and Stickland (1933) reported that adaptation occurred with formate and tryptic broth, it may be concluded that the tryptic broth contained carbohydrate or other sources of energy.

It appears that the majority of the amino acids used as nitrogenous supplements exerts a direct and specific action on hydrogenlyase adaptation. First, the experiments are of short duration and uncomplicated by cell growth, which confines the nutritional effects to the enzyme forming system. Secondly, most of the amino acids have a variety of pronounced effects of different magnitudes, whereas NH_3 in contrast is inactive. Thus, the cell may be completely or partially dependent on the essential or the stimulatory amino acids, respectively, and may be self sufficient or inert towards the inactive ones. Furthermore, the inhibitory amino acids may function as competitive homologues for crucial sites in the enzyme forming system and, thus, indirectly indicate adaptive requirements for the amino acids they antagonize. If it were necessary for the large number of active amino acids to undergo appreciable modification before participating in the adaptive process, one would expect the nature and the degree of their action to be less specific.

Billen and Lichstein (1951) have reported that a combination of six amino acids, e.g., methionine, cystine, valine, glutamic acid, tyrosine, and lysine, is almost as effective as hydrolyzed casein in inducing hydrogenlyase formation in *E. coli* during growth in a glucose salts basal medium. Also glutamic acid alone

stimulated the formation of hydrogenlyase in both growing cultures and non-proliferating cell suspensions. Likewise, in our cell suspension experiments, 0.7 per cent glutamic acid stimulated hydrogenlyase formation but only to the extent of 15 per cent of that obtainable with hydrolyzed casein. Moreover, the amino acids which we found to be stimulatory with resting cell suspensions are different in most instances from those found by Billen and Lichstein to stimulate growing cells. Since *E. coli* usually can synthesize all of its protein from NH_3, it is capable of extensive amino acid synthesis and interconversion. Therefore, it is not surprising that Billen and Lichstein's results with growing cultures do not parallel our results with resting cell suspensions. However, it seems reasonable to believe that our results indicate more directly the effect of amino acids on the production of formic hydrogenlyase.

The specificity of the amino acids for formic hydrogenlyase adaptation is supported by the absence of any amino acid effect on glucozymase, amino acid decarboxylases, and formic dehydrogenase. The concurrent increase in hydrogenase activity during formic hydrogenlyase adaptation can be regarded as an incidental adaptation to a product of formic hydrogenlyase activity, i.e., hydrogen, or simultaneous adaptation. In the latter case it would be necessary for hydrogenase to be part of the formic hydrogenlyase system. If this were true, the increase in hydrogenase activity still would not explain formic hydrogenlyase adaptation, since appreciable amounts of hydrogenase are present in the unadapted cell, whereas no formic hydrogenlyase can be detected even with higher concentrations of cells and longer time periods than used in our standard procedure. Furthermore, during adaptation the increase in hydrogenase is much less than that of formic hydrogenlyase. Therefore, much of the adaptive activity must be attributed to the formation of formic hydrogenlyase *per se* or at least of an intermediary enzyme distinct from formic dehydrogenase or hydrogenase.

Necessity for an energy source for adaptation and the inhibitory action of assimilatory poisons support the interpretation that synthesis is taking place during adaptation. It is highly possible that the amino acids are incorporated or compete in the synthesis of the hydrogenlyase enzyme. It has been established that enzyme adaptation involves some protein synthesis (Spiegelman, 1951; Pollock and Wainwright, 1948; Jebb, Knox, and Tomlinson, 1950; Billen and Lichstein, 1951), but the extent and the directness of this synthesis still are not established fully. In our experiments, the complex, intimate, and indispensable action of so many amino acids on adaptation suggests that a large part of the enzyme is synthesized from these simple units.

The inhibitory effect of NO_3^- and NO_2^- on formic hydrogenlyase activity was noted by Stephenson and Stickland (1932). In contrast to our results they failed to obtain complete inhibition of H_2 production even with as much as M/60 NO_3^-. This may have been due to high nitrite reductase activity of their cells which would rapidly destroy the toxic NO_2^- formed. In line with our results, Pakes and Jollyman (1901) found that 1 per cent KNO_3 completely prevented the formation of H_2 in formate and glucose broth cultures of coliform bacteria, Tubiash (1951) observed that NO_3^- and NO_2^- below 100 ppm N usually completely sup-

pressed gas formation in 48 hour sugar broth cultures of coliform organisms, and Yudkin (1932) reported almost complete or complete repression of hydrogenlyase formation by NO_3^- in cultures of *Escherichia freundii*.

Recently, Billen (1951) found that the formation of formic hydrogenlyase, hydrogenase, and formic dehydrogenase in growing cultures of *E. coli* was inhibited by differing concentrations of NO_3^-, and that high concentrations of amino acids reversed this inhibition. It was suggested that the synthesis of nitratase competed successfully against the synthesis of the other enzymes in a manner similar to the competitive enzyme interaction observed by Spiegelman and Dunn (1947). In our experiments NO_3^- and NO_2^- were toxic also for the activity of preformed formic hydrogenlyase and hydrogenase in the same relative order of enzyme sensitivity and at similar concentrations of the inhibitors as observed by Billen for the synthesis of these enzymes. This is suggestive of a more direct toxicity of NO_3^- and NO_2^- on adaptation as well as activity. The reversal of NO_3^- inhibition by high amino acid concentrations in the growth experiments could equally well be due to replacement of critical amino acids for hydrogenase and hydrogenlyase formation exhausted by degradation rather than by nitratase formation.

SUMMARY

Both a source of energy and amino acids are necessary for formic hydrogenlyase adaptation by nonproliferating suspensions of *Escherichia coli*. Among the amino acids, arginine, aspartic acid, and glutamic acid are essential, four others are stimulatory, and six are inhibitory for hydrogenlyase adaptation. The action of the amino acids appears to be direct and specific. It is suggested that formic hydrogenlyase adaptation involves extensive synthesis of the enzyme from amino acids.

Assimilatory poisons, oxygen, nitrate, and nitrite inhibit the formation of formic hydrogenlyase, and the latter two substances also inhibit the activity of the enzyme.

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