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Induced Biosynthesis of Formic Hydrogenlyase in Iron-Deficient Cells of *Escherichia coli*

T. FUKUYAMA[1] AND E. J. ORDAL
Department of Microbiology, University of Washington, Seattle, Washington

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ABSTRACT

FUKUYAMA, T. (University of Washington, Seattle), AND E. J. ORDAL. Induced biosynthesis of formic hydrogenlyase in iron-deficient cells of *Escherichia coli*. J. Bacteriol. **90**:673-680. 1965.—*Escherichia coli* cells were grown aerobically on a lactate-mineral salts medium from which iron had been removed by extraction with 8-hydroxyquinoline and chloroform. These cells carried out induced biosynthesis of formic hydrogenlyase in a reaction mixture containing glucose, formate, and phosphate without the addition of amino acids, providing adequate amounts of iron salts were present. In the absence of iron, glucose was fermented and acids were produced, but no formic hydrogenlyase developed. When iron-deficient *E. coli* cells were repeatedly washed, the property of carrying out induced biosynthesis of formic hydrogenlyase with glucose, formate, phosphate, and iron was lost, but was restored on addition of acid-hydrolyzed casein to the reaction mixture. An energy source (provided as glucose) was necessary for enzyme production. Iron-deficient cells were devoid of hydrogenase and formic hydrogenlyase but showed formic dehydrogenase activity when adequate amounts of selenium and molybdenum were present in the growth medium. Hydrogenase was consistently absent in iron-deficient cells but appeared concomitantly with formic hydrogenlyase during induced biosynthesis of the latter in iron-deficient cells of *E. coli*.

The decomposition of formate to carbon dioxide and molecular hydrogen is due to an enzyme system given the name formic hydrogenlyase by Stephenson and Stickland (1932). There has been a considerable amount of controversy as to whether formic hydrogenlyase, sometimes referred to as hydrogenlyase, is a single distinct enzyme or a multi-enzyme system which includes the enzymes hydrogenase and formic dehydrogenase (Gest, 1954). The mechanism of the formic hydrogenlyase reaction was investigated by Gest and Peck (1955) and Peck and Gest (1957), and evidence was presented that formate decomposition to hydrogen and carbon dioxide is catalyzed by a multi-enzyme system consisting of a formic dehydrogenase, hydrogenase, and one or two intermediate factors involved in electron transport. However, there is still not complete agreement as to the nature of formic hydrogenlyase, and, as pointed out by Upadhyay and Stokes (1963*a*): "There is considerable, but not conclusive, evidence that the decomposition of formic acid into H_2 and CO_2 by formic hydrogenlyase may involve the participation of two additional enzymes, hydrogenase and formic dehydrogenase."

The inducible character of formic hydrogenlyase was first recognized by Stephenson and Stickland (1933), who reported that production of the enzyme occurred in cell suspensions of *Escherichia coli* without appreciable cell multiplication when cells were incubated anaerobically with formate and tryptic broth. Subsequently, Pinsky and Stokes (1952) and Kushner and Quastel (1953) showed that induced biosynthesis of formic hydrogenlyase in nonproliferating cells of enteric bacteria was dependent upon an energy source and availability of an external supply of amino acids, as well as formate as an inducer. In a recent study, Upadhyay and Stokes (1963*b*) showed that formic hydrogenlyase could be induced in resting-cell suspensions of an unnamed psychrophilic bacterium, if glucose, formate, and casein hydrolysate were provided as supplements, though several times as much glucose was required as for enzyme induction in strains of *E. coli* and *Salmonella*. Formic hydrogenlyase is absent in cultures grown with vigorous aeration (Gest, 1954), and Pichinoty (1962) has shown that this is due to oxygen repression of the biosynthesis of the enzyme system.

Involvement of iron in formic hydrogenlyase

[1] Present address: Department of Bacteriology, Medical School, University of Southern California, Los Angeles.

activity was suggested in an early study by Waring and Werkman (1944), who showed that formic hydrogenlyase, formic dehydrogenase, and hydrogenase activities in *Aerobacter indologenes* were markedly suppressed by iron deficiency. Gest and Gibbs (1952) showed that, not only was formic hydrogenlyase activity inhibited by α,α'-dipyridyl, but that this inhibition was relieved by ferrous iron. Definite proof of the presence of iron in hydrogenase was obtained by Whiteley and Ordal (1956).

Studies in this laboratory on the role of iron in formate decomposition were initiated to obtain further evidence of the roles of hydrogenase and formic dehydrogenase in formate decomposition, and with the objective of developing methods for studying the unidentified components or cofactors presumably operating in conjunction with hydrogenase and formic dehydrogenase in the reversible conversion of formate to carbon dioxide and hydrogen.

In some preliminary experiments, it was found that suspensions of *E. coli* cells grown aerobically on an iron-deficient synthetic basal medium were completely devoid of hydrogenase and formic hydrogenlyase activity, although they showed moderate formic dehydrogenase activity. Yet, suspensions of these cells were found to carry out induced biosynthesis of formic hydrogenlyase without the addition of amino acids. This result was somewhat surprising in view of reports by Pinsky and Stokes (1952), Kushner and Quastel (1953), and Upadhyay and Stokes (1963*b*) that the addition of amino acids or casein hydrolysate was required for induced biosynthesis of formic hydrogenlyase in resting-cell suspensions of *E. coli*. It was also found that induced biosynthesis of formic hydrogenlyase was dependent upon the presence of iron. Further studies have now been carried out, and our findings are presented in this report.

MATERIALS AND METHODS

The growth medium normally employed in this investigation contained the following components: sodium lactate, 1.0 g; K_2HPO_4, 0.4 g; KH_2PO_4, 0.1 g; $(NH_4)_2SO_4$, 0.1 g; $MgSO_4 \cdot 7H_2O$, 0.01 g; and 100 ml of glass-distilled water. In a few experiments, the concentration of phosphate was increased two to three times. The various components of the medium were rendered iron-deficient by extraction with 8-hydroxyquinoline and chloroform in an adaptation of the method used by Waring and Werkman (1942). For reasons indicated in the text, 0.5 to 1.0 μg of selenium and molybdenum as selenate and molybdate, respectively, were added to each flask of the growth medium with some batches of reagents used for culture media.

Stock cultures of *E. coli*, University of Washington strain 325, were maintained on nutrient agar slants. Iron-deficient suspensions of cells were obtained by inoculating 500-ml Florence or Erlenmeyer flasks containing 100 to 150 ml of iron-deficient medium with 1 to 1.5 ml of a previously grown iron-deficient culture. The cultures were incubated aerobically on a shaker at 35 C. After 16 hr, the cells were harvested by centrifugation, washed once with iron-deficient 0.01 M phosphate buffer (*p*H 7), centrifuged, and suspended in phosphate buffer of the same composition to give approximately a 10% cell suspension by volume.

The initial *p*H of the medium was 6.9, and when growth ceased in iron-deficient media no change in *p*H had occurred, since lactate had been oxidized to acetate. It was determined in a number of experiments that at 16 hr cultures had reached the stationary stage owing to iron deficiency. For comparative purposes, some suspensions of *E. coli* were also prepared from cultures grown on a normal medium, i.e., the synthetic basal medium without the removal of iron.

To prepare cell-free extracts, suspensions of *E. coli* cells were disrupted with a Bronson LS 75 sonifier with a 0.5-inch tip. Effective disruption occurred in around 2 min when 4 to 6 ml of a suspension of cells containing 4 to 5 mg (dry weight) of cells per ml were sonically treated. Subsequent centrifugation at 5,000 $\times$ *g* for 15 min in a small volume in a Servall centrifuge removed the majority of the remaining intact cells.

Hydrogenase, formic dehydrogenase, and formic hydrogenlyase activities were determined in the Warburg apparatus at 37 C by means of conventional procedures. Hydrogenase activity was measured in terms of hydrogen uptake in a hydrogen atmosphere with 0.2 ml of 0.037 M methylene blue as acceptor in a phosphate buffer at *p*H 6.2. Formic dehydrogenase activity was measured in terms of CO_2 evolution from 0.2 ml of 0.25 M sodium formate in a nitrogen atmosphere at *p*H 6.2 with methylene blue as oxidizing agent. Formic hydrogenlyase activity was measured in terms of hydrogen evolution from formate at *p*H 7.0 with 0.2 ml of 0.25 M sodium formate as substrate in a nitrogen atmosphere with 0.2 ml of 15% KOH in the center well. A 1-ml amount of 0.066 M phosphate buffer at the appropriate *p*H, and 0.2 to 0.5 ml of a cell suspension, were used in each Warburg cup in a total volume of 2.0 ml. In a number of experiments, it was determined that the concentration of cells employed ranged from 2 to 5.5 mg (dry weight) per ml. All reagents except methylene blue were treated with 8-hydroxyquinoline and chloroform to remove iron.

When induced biosynthesis of formic hydrogenlyase was studied, 0.2 ml of a 0.10 M glucose solution and 0.1 ml of a solution containing 4.0 μg of Fe^{++} per ml were added in addition to the other reagents used in determination of formic hydrogenlyase activity.

To make a simple comparison of the relative effects of iron and glucose on hydrogenlyase activ-

ity, the data from two experiments are plotted in terms of Q_{H_2} values, here designated as the number of microliters of H_2 evolved per hour per milligram of dry cells. The actual points plotted represent one-half the microliters of H_2 evolved per milligram of dry cells in an experiment lasting for 2 hr.

RESULTS

Requirement for iron in induced biosynthesis of formic hydrogenlyase by iron-deficient suspensions of E. coli. The requirement for iron in the induced biosynthesis of formic hydrogenlyase by iron-deficient cells of *E. coli* is shown in the experiment illustrated in Fig. 1. All reagents had been treated to remove iron. It may be noted that no hydrogen was produced from formate or from formate and iron. Hence, the iron-deficient cells must be considered to be completely devoid of formic hydrogenlyase activity. Cell-free extracts of iron-deficient cells were also found to be devoid of formic hydrogenlyase activity. This is not surprising, since the cells were grown aerobically by oxidation of a substrate, and Pichinoty (1962) has shown that oxygen represses biosynthesis of formic hydrogenlyase. In the absence of an energy source, biosynthesis of formic hydrogenlyase did not occur. On the other hand, when glucose and ferrous iron in addition to formate were added to the Warburg vessels, active hydrogen production began after an induction period of about 20 to 30 min, indicating that enzymatic adaptation had taken place. In the absence of added iron, a small amount of hydrogen was produced from glucose and formate, indicating that some formic hydrogenlyase activity had developed. In some experiments in which the same procedures were employed, the evolution of hydrogen from glucose and formate without added iron was considerably greater than that in the experiment illustrated in Fig. 1. In other cases, it was essentially equivalent. Much of this variation was most likely due to the presence of residual iron in the reagents employed in various experiments or to the contamination of the reagents or Warburg vessels with iron. In spite of elaborate precautions, such contamination did occur. Thus, in one experiment where unexpectedly large amounts of hydrogen were produced from formate and glucose without addition of iron, it was found that one of the reagents had been contaminated with enough iron to be detectable with 8-hydroxyquinoline and chloroform or 1–10 *o*-phenanthroline.

Iron-deficient cells of *E. coli* grown under the conditions described in this paper normally exhibited a moderate formic dehydrogenase activity. However, when some batches of reagents were employed, formic dehydrogenase activity

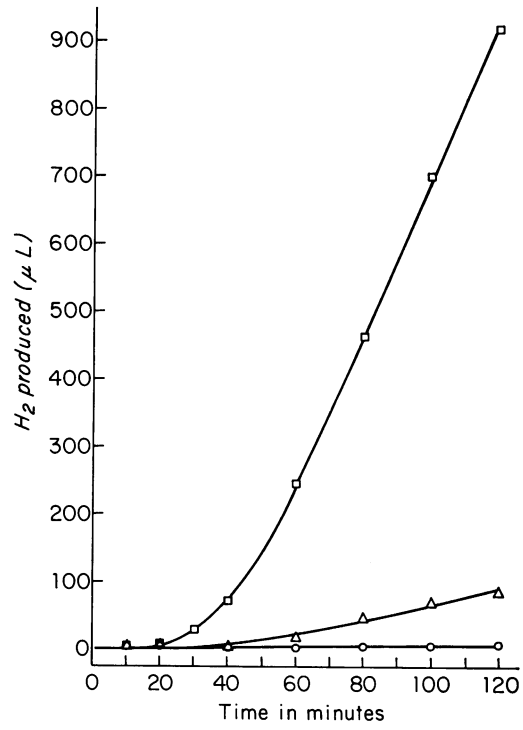

FIG. 1. *Effect of iron and glucose on the induced biosynthesis of formic hydrogenlyase by iron-deficient suspensions of cells of Escherichia coli. Symbols:* ○ = *formate, formate and* Fe^{++}; △ = *formate and glucose;* □ = *formate, glucose, and* Fe^{++}.

was low or even absent. Formic dehydrogenase activity increased when a growth medium made up from such reagents was fortified by addition of small amounts of selenite and molybdate (Fig. 2). This finding confirms the report by Pinsent (1954) that trace amounts of selenium and molybdenum are required for the formation of active formic dehydrogenase by strains of coliform bacteria grown in a purified synthetic medium.

Hydrogenase was found to be absent in iron-deficient cells and cell-free extracts of *E. coli*. However, after induced biosynthesis of formic hydrogenlyase in the presence of glucose, formate, and iron, hydrogenase activity was always found present in cells and cell-free extracts. Apparently, biosynthesis of hydrogenase occurred concomitantly with biosynthesis of formic hydrogenlyase. This would, of course, be expected if hydrogenase is involved in formic hydrogenlyase activity as part of a multi-enzyme complex.

Since intact cells were employed in the experiment cited above, it was necessary to consider whether these iron-deficient cells were freely

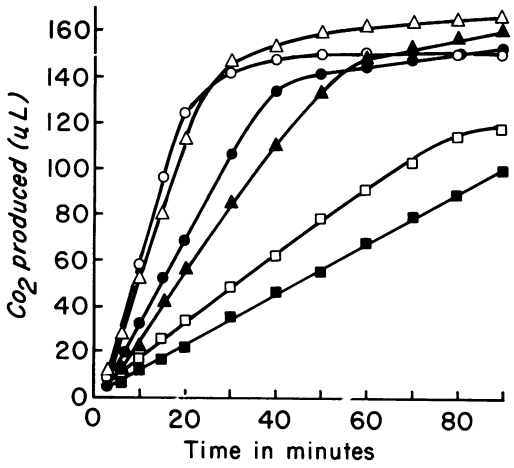

FIG. 2. *Formic dehydrogenase activity in cells and cell-free extracts of iron-deficient Escherichia coli. With 1.0 μg of Se and 1.0 μg of Mo per 150 ml added to culture medium:* ○ = *cells [1.5 mg (dry weight) per ml];* △ = *cell-free extract;* ● = *cells (one-half concentration);* ▲ = *cell-free extract (one-half concentration). No Se or Mo added to culture medium:* □ = *cells [1.5 mg (dry weight) per ml];* ■ = *extract.*

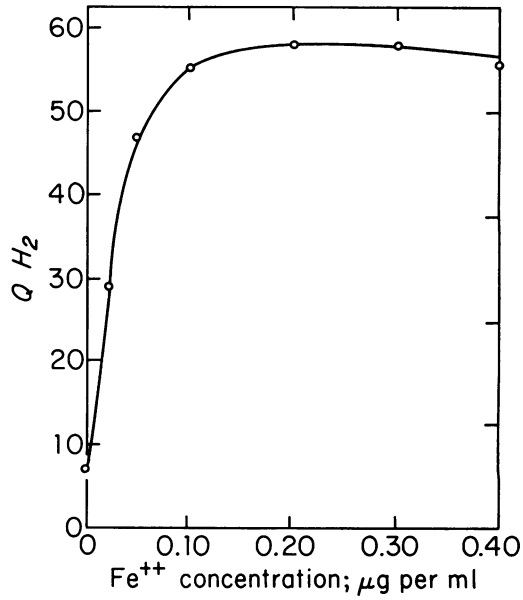

FIG. 3. *Effect of concentration of iron on induced biosynthesis of formic hydrogenlyase in suspensions of iron-deficient cells of Escherichia coli.*

permeable to the substrates employed. Barrett, Larson, and Kallio (1953), Cohen and Monod (1957), Englesberg, Watson, and Hoffee (1961), and others have considered the problem of transport across cell membranes and demonstrated that there may be specific permeability barriers to utilization of organic acids and some carbohydrates by bacteria. Hence, it appeared logical to determine whether the lag in production of formic hydrogenlyase by iron-deficient cells might be due to induction of a formate transport system. Bovell, Packer, and Helgerson (1963) reported that resting cells of *E. coli* strain B grown either on Penassay Broth (Difco) or on a glucose-mineral salts medium were freely permeable to sodium formate. That iron-deficient cells of *E. coli* strain 325 grown on a lactate-mineral salts medium are also freely permeable to formate is shown in Fig. 2, in which the formic dehydrogenase activities of intact cells are compared with those of cell-free extracts prepared from a sample of the cell suspension. Immediate evolution of CO_2 occurred with both cell suspensions and cell-free extracts, indicating that there was no barrier to penetration of formate.

After induction of formic hydrogenlyase in iron-deficient cells, these cells or cell-free extracts prepared from them showed immediate production of hydrogen from formate; consequently, induced cells must also be considered to be freely permeable to formate.

The influence of the concentration of ferrous iron on the synthesis of formic hydrogenlyase is shown in Fig. 3. The relative amounts of hydrogen produced per hour per milligram (dry weight) of cells, here indicated as Q_{H_2}, are plotted against the concentration of iron. Maximal formic hydrogenlyase activity was obtained on the addition of 0.2 μg of Fe^{++} per ml of Warburg cup contents. The sensitivity of the system to added iron is clearly shown by the fact that a considerable degree of activity developed with addition of 0.05 μg of Fe^{++} per ml of Warburg cup contents. From the shape of the curve in Fig. 3, the effect of small amounts of residual or contaminating iron in the reagents is self-evident.

The requirement for iron for induced biosynthesis of formic hydrogenlyase was specific. Although Fe^{+++} could replace Fe^{++}, other inorganic ions such as Co^{++}, Zn^{++}, Mn^{++}, and Cu^{++} were without effect.

Effect of chloramphenicol. One possible explanation for the lack of an amino acid requirement for production of formic hydrogenlyase in iron-deficient cells is that formic hydrogenlyase is in fact produced during growth but is inactive owing to a lack of iron. In this hypothesis, glucose is needed because energy is required to link the iron to the enzyme or enzyme system. Hence, it is necessary to test this hypothesis and to deter-

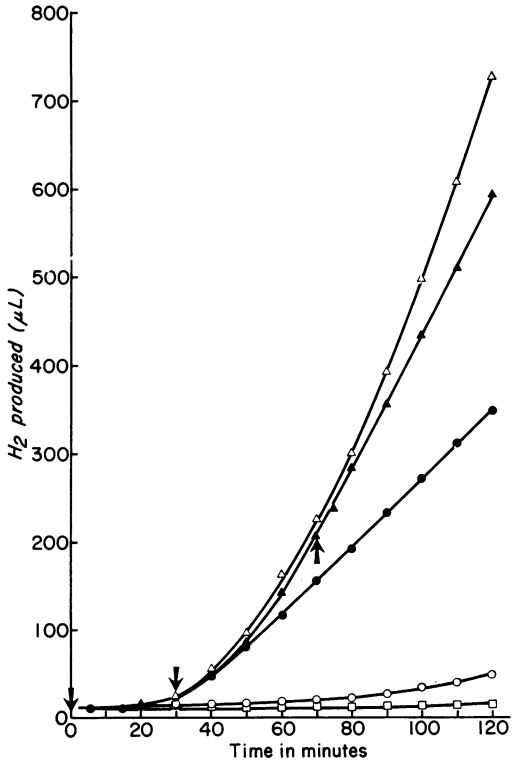

FIG. 4. *Effect of chloramphenicol on biosynthesis of formic hydrogenlyase in iron-deficient cells of Escherichia coli. Symbols:* ○ *= 0.0 μg of Fe per ml;* △ *= 0.5 μg of Fe per ml;* □ *= 0.5 μg of Fe per ml and 25 μg of chloramphenicol (CAP) at zero-time;* ● *= 0.5 μg of Fe per ml and 25 μg of CAP at 30 min;* ▲ *= 0.5 μg of Fe per ml and 25 μg of CAP at 70 min.*

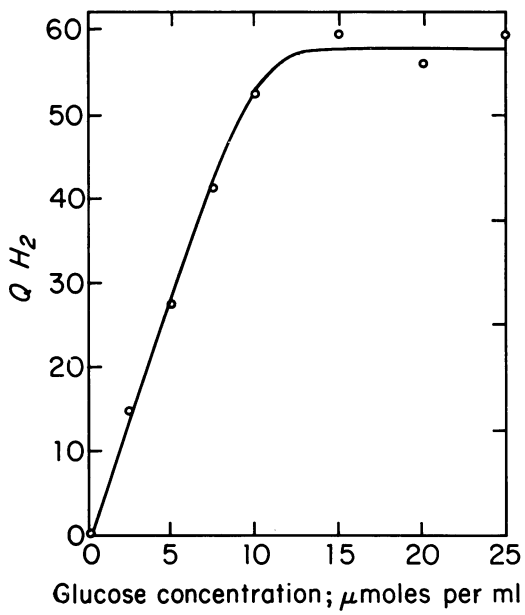

FIG. 5. *Effect of concentration of glucose on induced biosynthesis of formic hydrogenlyase in suspensions of iron-deficient cells of Escherichia coli.*

mine whether de novo protein synthesis is a requirement for production of formic hydrogenlyase in iron-deficient cells of *E. coli*. In the experiment shown in Fig. 4, it is seen that chloramphenicol completely blocks biosynthesis of formic hydrogenlyase when added at zero-time, though there is little or no effect on existing formic hydrogenlyase, as judged by the rate of hydrogen production on addition of chloramphenicol at 70 min. Hence, it may be concluded that protein synthesis is a prerequisite for formic hydrogenlyase production, even though an external supply of amino acids is not needed by iron-deficient cells of *E. coli*.

Energy requirement for induced biosynthesis of formic hydrogenlyase by iron-deficient cells of E. coli. As was shown in Fig. 1, no hydrogen was produced from formate or formate plus iron in the absence of glucose. It was repeatedly found that formic hydrogenlyase activity never developed in iron-deficient cells incubated with formate or with formate plus iron. On the other hand, with reaction mixtures containing glucose in addition to formate and iron, enzyme activity regularly and characteristically appeared after an induction period. In line with the findings of other investigators (Pinsky and Stokes, 1952; Kushner and Quastel, 1953; Upadhyay and Stokes, 1963*b*), it must be concluded that energy for the synthesis of the enzyme is provided by fermentation of glucose. The effect of the concentration of glucose in the reaction mixture containing formate and ferrous iron is shown in Fig. 5. From this experiment, it may be seen that the formic hydrogenlyase activity which developed was essentially proportional to the concentration of glucose, with maximal activity obtained at a level of approximately 12 μmoles of glucose per ml of reaction mixture or 24 μmoles per Warburg cup. This value is somewhat more than the value of 17 μmoles of glucose per Warburg cup reported by Upadhyay and Stokes (1963*b*) as being required for maximal induction of formic hydrogenlyase in resting cells of *E. coli* supplemented with casein hydrolysate and formate, but it is less than the amount (55 μmoles of glucose) required by cells of a psychrophilic bacterium.

When glucose alone was used as substrate in the presence of ferrous iron, evolution of hydro-

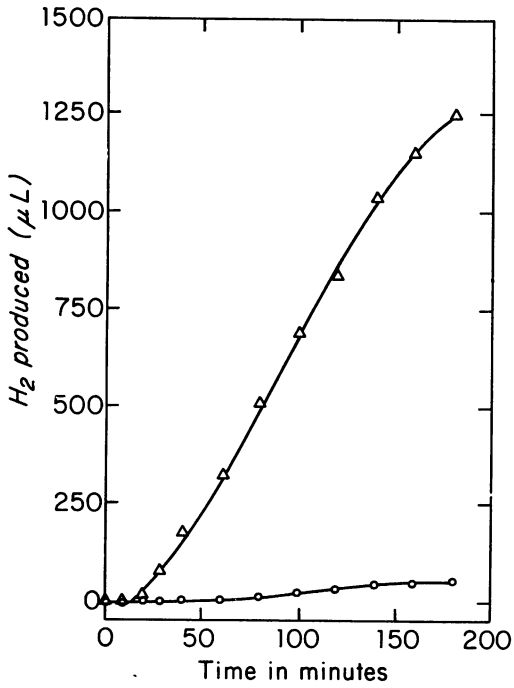

FIG. 6. *Effect of casein hydrolysate on the induced biosynthesis of formic hydrogenlyase in suspensions of cells of Escherichia coli grown on normal synthetic medium (not iron-deficient). Symbols: ○ = no casein hydrolysate; △ = 0.5% casein hydrolysate.*

gen was regularly observed after a characteristic induction period, though hydrogen evolution was always materially less than when formate was also initially present in the reaction mixture. It is well known that formate is produced as an intermediate in fermentation of sugars by *E. coli*, and, until formic hydrogenlyase activity develops, there should be an increasing concentration of formate which serves as inducer. As might be expected, the presence of formate in the initial reaction mixture leads to increased formic hydrogenlyase activity.

When iron as well as formate was omitted from the reaction mixture, little or no hydrogen was ordinarily produced from glucose, though it was demonstrated by analysis of reducing sugars that glucose was utilized. This finding is in agreement with that of Waring and Werkman (1944), who reported that no production of hydrogen occurred when glucose was fermented by iron-deficient cells of *A. indologenes*, although considerable amounts of formic and lactic acids were produced.

Amino acid requirement. Suspensions of *E. coli* cells grown on normal lactate-mineral salts medium, i.e., medium which had not been treated with 8-hydroxyquinoline and chloroform, failed to carry out induced biosynthesis of formic hydrogenlyase unless a source of amino acids was provided. An experiment of this type is illustrated in Fig. 6, where it is shown that the addition of 0.5% casein hydrolysate leads to synthesis of formic hydrogenlyase in the presence of glucose, formate, and iron, whereas in the absence of casein hydrolysate essentially no formic hydrogenlyase was produced. Hence, cells of *E. coli* grown aerobically on normal lactate-mineral salts behaved like the suspensions of *E. coli* which had been grown aerobically on organic media (Pinsky and Stokes, 1952; Kushner and Quastel, 1953; Upadhyay and Stokes, 1963*b*).

Since the addition of casein hydrolysate or amino acids was not necessary for induced biosynthesis of formic hydrogenlyase with iron-deficient cells, it was logical to believe that these latter cells contained an internal supply of nitrogenous components utilizable for enzyme synthesis. When iron-deficient cells of *E. coli* were subjected to repeated and rigorous washings, the capacity to carry out induced biosynthesis of formic hydrogenlyase in reaction mixtures containing glucose, formate, and ferrous iron was lost. However, this property was restored and, in fact, improved by addition of acid-hydrolyzed casein (Table 1). Data from this experiment suggest that amino acids are available for protein synthesis in iron-deficient cells of *E. coli* but that these are leached out during the process of washing. Although Taylor (1947) reported that free amino acid pools were not present in gram-negative bacteria, it was shown by Proom and Woiwood (1949), Britten and McClure (1962), and other investigators that free amino acid pools did exist in suspensions of *E. coli* cells. The presence of a free amino acid pool in iron-deficient cells of *E. coli* was confirmed by paper chromatography according to the method of Proom and Woiwood (1949). Easily recognizable amounts of glutamic acid, glycine, aminobutyric acid, and lysine, with traces of other amino acids, were found present in iron-deficient cells of *E. coli*. It is tempting to ascribe the capability of induced

TABLE 1. *Effect of casein hydrolysate on induced biosynthesis of formic hydrogenlyase by washed cells of iron-deficient Escherichia coli*

No. of washings	Q_{H_2}	
	With no addition	With 0.5% casein hydrolysate
1	68	87
3	14	81
6	0	73

biosynthesis of formic hydrogenlyase to the presence of a free amino acid pool in iron-deficient cells of *E. coli*, but insufficient work has been done to justify this conclusion. It is not yet possible to account for the fact that resting cells of iron-deficient bacteria can carry out induced biosynthesis of formic hydrogenlyase in the absence of casein hydrolysate, whereas cells grown in complete media (without the extraction of iron) and cells grown on complex organic media are unable to do so. Further investigation is required to settle this question.

DISCUSSION

The necessity for the presence of amino acids in the reaction mixture to obtain induced biosynthesis of formic hydrogenlyase in resting cells of *E. coli* grown on organic media has been documented by a number of investigators, most recently by Upadhyay and Stokes (1963*b*). Billen and Lichstein (1951) reported that cells of *E. coli* harvested from a glucose-mineral salts medium contain no measurable formic hydrogenlyase activity. However, when casein hydrolysate or smaller sets of amino acids were added to growing cultures, active formic hydrogenlyase appeared, although the amino acids were not required for growth. In the present investigation, it was found that suspensions of *E. coli* cells grown on lactate-mineral salts medium which was not extracted to remove iron failed to carry out induced biosynthesis of formic hydrogenlyase unless a set of amino acids, such as that in acid-hydrolyzed casein, was added. This finding was in complete agreement with those reported in the literature (Upadhyay and Stokes, 1963*b*).

In sharp contrast was the discovery that suspensions of iron-deficient cells of *E. coli* could carry out induced biosynthesis of formic hydrogenlyase in the presence of glucose and formate without the addition of amino acids, provided adequate amounts of iron salts were present. In the absence of iron, no hydrogen was produced, and glucose was fermented with production of acids, confirming the report of Waring and Werkman (1944) with *A. indologenes*.

When iron-deficient cells of *E. coli* were repeatedly washed, the property of carrying out induced biosynthesis of hydrogenlyase was lost, but was regained on addition of acid-hydrolyzed casein to the reaction mixture. It is possible that this behavior is due to the leaching out of a free amino acid pool, since it is now well documented that a free amino acid pool exists in *E. coli* (Britten and McClure, 1962). However, such an explanation may not be adequate, since Mandelstam (1960) has shown that a considerable protein turnover occurs in nongrowing cells of *E. coli*. This point requires further investigation.

The requirement of added amino acids for induced biosynthesis of formic hydrogenlyase shown by cells of *E. coli* grown on complex media (Upadhyay and Stokes, 1963*b*) and the absence of this requirement in iron-deficient cells has not yet been accounted for. The possibility that this may be accounted for by deficiencies in inorganic elements is being actively explored.

The iron-deficient cells employed in this study were initially completely devoid of hydrogenase and formic hydrogenlyase but contained a definite formic dehydrogenase activity. When some batches of reagents were used in culture media, formic dehydrogenase activity was absent or low, unless small amounts of selenite and molybdate were added to the culture medium. This confirmed the report by Pinsent (1954) that the presence of selenium and molybdenum in the medium was necessary for synthesis of formic dehydrogenase during growth. After induced biosynthesis of formic hydrogenlyase had taken place in iron-deficient cells of *E. coli*, it was found that the cells now contained hydrogenase activity as well. This is one more link in the chain of evidence which supports the view of Peck and Gest (1957) that hydrogenase is a component of the enzyme system responsible for the production of hydrogen and carbon dioxide from formate.

The necessity for an energy source, here provided in the form of glucose, for induced biosynthesis of formic hydrogenlyase was found to be explicit. In the absence of iron, the enzyme was not produced, though fermentation of glucose without hydrogen production occurred as reported by Waring and Werkman (1944). The requirement of an energy source has been well documented by other investigators. It is of interest that the concentration of glucose required for induced biosynthesis of formic hydrogenlyase by iron-deficient cells of *E. coli* in the absence of added amino acids was greater than that shown to be necessary for synthesis of the same enzyme when amino acids in the form of casein hydrolysate were provided (Upadhyay and Stokes, 1963*b*).

Iron has been found to be a requirement for the biosynthesis of formic hydrogenlyase, and of hydrogenase which appears at the same time in iron-deficient cells of *E. coli*. Hence, the system which has been developed provides a means for incorporation of radioactive iron during short-time biosynthesis of the formic hydrogenlyase complex. It might be expected that when bacteria are grown aerobically with limiting iron the available iron would be incorporated into systems such as the cytochromes which are needed for aerobic growth. Since induced biosynthesis of

formic hydrogenlyase in iron-deficient cells does not occur unless an external source of iron is supplied, it may be concluded that existing iron in the cells is tightly bound, as would be the case with iron present in the cytochromes. It might also be expected that, if a limiting amount of radioactive iron were supplied during short-time induced biosynthesis of enzymes, this iron would go primarily into the enzyme systems developing under anaerobic conditions. Among these systems would be the formic hydrogenlyase complex, including the enzyme hydrogenase and the cofactors postulated by Peck and Gest (1957). In particular, if iron is present in these cofactors, labeling with radioactive iron should facilitate their separation and identification.

Preliminary experiments have shown that when limiting amounts of radioactive iron were added during induced biosynthesis of formic hydrogenlyase, there was almost complete absorption of the radioactive iron by the cell suspension. Studies are now aimed at discovering the locations as well as functions of this radioactive iron in cells of *E. coli*.

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