Factors Underlying Bacterial Enzyme Synthesis.* (20125)

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Although it has long been known that *E. coli* utilizes ammonia for growth, and, therefore, synthesizes its protein requirements from this substance, the adaptive formation of formic hydrogenlyase in this organism seems only to take place in the presence of certain amino acids. Billen and Lichstein(1) have shown that the formation of this enzyme in growing cells is stimulated by several amino acids, the most important of these being L-glutamate and DL-methionine. Formation of the enzyme also takes place if resting cells are incubated in the presence of glucose and L-glutamate for 6 hours. Pinsky and Stokes(2) have pointed out that both a source of energy and certain amino acids are required for the development of the enzyme in resting cells of *E. coli*. They find that L-arginine, L-aspartic acid and L-glutamic acid are extremely important for synthesis, that L-cystine, glycine, DL-serine, and DL-threonine have stimulatory effects, whilst other amino acids including L-cysteine, L-leucine, and DL-methionine are inhibitory.

In this paper, studies are reported of the means by which energy for formic hydrogenlyase synthesis may be obtained and of the nitrogen sources used in formic hydrogenlyase synthesis by 2 strains of *E. coli*.

Methods. The strains of *E. coli* used in the present investigations were isolated from feces (Strain 1), and from pus (Strain 2). Both were maintained on nutrient agar at 37° C. Twelve to 18 hours cultures were used, substantially the same results being obtained with these as with younger (5 to 8 hour) cultures. The bacteria were scraped off the agar surface, washed 3 times in distilled water, and a bacterial suspension was made up to contain 3 mg (dry weight) per ml. The Warburg manometric apparatus was used for determination of enzymic activity. Each manometric vessel contained 0.5 ml of 0.2 *M* sodium phosphate buffer (pH 7.4, unless otherwise stated), 0.2 ml of *M* sodium formate or *M* glycerol, or 0.5 *M* glucose in a final volume of 3.0 ml and other substances as specified. 0.5 ml of the bacterial suspension was placed in the side arm, and filter paper, moistened with KOH, was inserted in the center well to absorb liberated carbon dioxide. After gassing for 15 minutes with nitrogen, and equilibration, the bacterial suspension was tipped into the main vessel. All experiments were carried out at 37° C. After the addition of the bacteria to the solutions in the main vessel, an interval of time always elapsed before the first appearance of hydrogen (indicating the presence of formic hydrogenlyase) was observed, and a further period of time was required before a constant rate of hydrogen evolution was reached. These initial periods are termed Lag T_1 and Lag T_2, respectively, and the final enzyme activity is expressed as Q_{H_2}, this being the volume of hydrogen (cmm) evolved per mg dry weight bacteria per hour. For the experiments on preadapted cells, organisms were used which had been grown overnight in a broth containing yeast extract, peptone and glucose, and which, therefore, possessed formic hydrogenlyase activity.

Results. Energy requirements for formic hydrogenlyase synthesis. Stephenson and Stickland(3) observed that formic hydrogenlyase synthesis takes place in the presence of formate and a nutrient tryptic broth. Pinsky and Stokes subsequently(2) found that such synthesis does not occur in the presence of formate and a casein hydrolysate unless glucose is also added. We have found that when adaptation is allowed to take place in the presence of sodium formate, and of bactopeptone as a source of nitrogen, a marked stimulation of the final rate of hydrogen evo-

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TABLE I. Effects of Sodium Salts of DL-Aspartate, Fumarate, and Nitrate on Hydrogen Production by *E. coli* from Formate (0.067*M*) or Glycerol (0.067*M*) in Presence of Peptone.

Strain of *E. coli*	System*	Lag, min. T_1	Lag, min. T_2	Final Q_{H_2}
1	F + 1% p	60	80	50
	F + 1% p + .1 *M* DL-aspartate	"	110	187
	F + 1% p + .1 *M* f	"	100	198
1	F + 1% p	40	80	70
	F + 1% p + .01 *M* f	"	80	368
1	F + 1% p	100	100	19
	F + 1% p + .01 *M* n	30	70	271
2	F + 1.67% p	"	90	221
	F + 1.67% p + .01 *M* f	"	70	328
	F + 1.67% p + .01 *M* n	"	40	307
1	G + 1% p	>180	—	0
	G + 1% p + .01 *M* f	160	170	52
1	1% p	>180	—	0
	1% p + .01 *M* f	> "	—	0
	1% p + .01 *M* n	> "	—	0

* F = formate; G = glycerol; p = peptone; f = fumarate; n = nitrate.

TABLE II. Effects of Sodium Fumarate, Sodium Nitrate, Ammonium Chloride and Amino Acids on the Formic Hydrogenlyase Activity of Adapted Cells.

Addition	Formic hydrogenlyase activity, % of control Strain 1	Strain 2
Nil	100	100
Dl-Serine	107	105
L-Histidine	98	100
L-Arginine	103	102
L-Leucine	103	104
DL-Aspartate	93	86
L-Lysine	—	103
DL-Valine	—	104
20 amino acids	102	103
.01 *M* Sodium fumarate	66	43
.01 *M* Sodium nitrate	58	41
.1 *M* Ammonium chloride	106	100

Amino acid concentrations are as shown in legend to Fig. 1. Enzymatic activity was estimated by measurement of the evolution of hydrogen from formate during a 20 min. period. The data in this table summarize the results of several experiments.

lution is brought about by the addition to the mixture of either DL-aspartate, fumarate or nitrate. The addition of succinate produces no effect. These effects are observed, with minor quantitative differences, with both the strains of *E. coli* that we have employed. Typical results are shown in Table I. None of these compounds stimulates the evolution of hydrogen by pre-adapted cells (Table II). Indeed, the presence of fumarate or nitrate diminishes the rate of evolution of hydrogen by these cells, probably because of their abilities to act as hydrogen acceptors. The observed stimulation on the enzymic activity of unadapted cells must presumably be due to an accelerative effect on the process of enzyme synthesis. It is known that formic hydrogenlyase formation in *E. coli* is not dependent upon cell proliferation(2,3), and turbidity measurements in some of our experiments have confirmed this fact. It was found also that the addition of nitrate to a formate-peptone medium produced a maximum effect on adaptation with less than 5% increase in turbidity. The work of Quastel *et al.*(4,5), has shown that the presence of L-aspartate, fumarate or nitrate will enable cells of *E. coli* to proliferate in the absence of oxygen. Our present results show that these substances will also serve as energy sources for enzyme synthesis in the absence of cell growth. The energy required for the increased rate of synthesis is derived from an anaerobic oxidation accomplished by fumarate or by the other hydrogen acceptors tested. It is of interest in this connection to recall the observation of Quastel and Wooldridge(6) that anaerobic growth of *E. coli* is far greater in a formate-fumarate medium than in a fumarate medium devoid of formate; the explanation advanced to account for this phenomenon was that extra energy became available through the oxidation of formate by fumarate. The effect of fumarate in enhancing the rate of synthesis of formic hydrogenlyase is also seen clearly when glycerol is present as hydrogen donor. Whilst a mixture of glycerol and peptone or a mixture of fumarate and peptone is ineffective in securing synthesis of the enzyme in *E. coli*, the addition of fumarate to a glycerol-peptone mixture brings about the synthesis (Table I).

Effects of DL-aspartate, and of ammonia, on synthesis of formic hydrogenlyase. It is likely that the presence of aspartate stimulates adaptation, owing to its conversion to fumarate in *E. coli* (Quastel and Woolf(7)). This conclusion is supported by the fact that the presence of ammonium ions markedly

TABLE III. Effect of Ammonium Chloride and Sodium Nitrate on Formic Hydrogenlyase Formation in *E. coli*.

Strain	System*	Lag, min. T_1	Lag, min. T_2	Final Q_{H_2}
1	G + 5 am†	50	80	592
	G + 5 am + .1 *M* ammonium Cl	50	80	774
	G + 5 am + .01 *M* sodium nitrate	>125	—	0
2	F + 1% p	40	90	97
	F + 1% p + .1 *M* ammonium Cl	80	80	12
1	F + 1.67% p	30	70	56
	F + 1.67% p + 0.1 *M* ammonium Cl	>120	—	0
	F + 1.67% p + .01 *M* sodium nitrate	30	70	368
	F + 1.67% p + .1 *M* ammonium Cl + .01 *M* sodium nitrate	30	70	110

* G = glucose; am = amino acids; F = formate; p = peptone.

† Dl-serine, L-histidine, L-arginine, L-leucine, and Dl-aspartate, as in Fig. 1.

affects formic hydrogenlyase synthesis. The presence of 0.1 *M* ammonium chloride strongly, or completely, inhibits formic hydrogenlyase formation in the presence of a mixture of formate and peptone but has no inhibitory effect in the presence of a mixture of glucose and amino acids (Table III). It is suggested that in a formate-peptone mixture, energy is supplied for adaptation by the transfer of hydrogen from a donor (formate or an amino acid) to fumarate, which arises by the action of aspartase from aspartate present in the peptone. The presence of ammonium ions shifts the equilibrium, L-aspartate $\rightleftarrows$ fumarate + NH_3, to the left, removing the fumarate and, therefore, inhibiting adaptation. In the presence of glucose, the energy liberated by glycolysis is available for adaptation, and the phenomenon may take place even in the presence of excess ammonium ions. Doubtless L-aspartate plays a role as hydrogen acceptor, after conversion to fumarate, even in the presence of glucose, and thus affects the rate of synthesis of the enzyme.

We have found that aspartate may be replaced by fumarate but not by succinate for formic hydrogenlyase synthesis. The possibility that the presence of L-aspartate may exert specific effects is not, of course, excluded by these results.

Effect of nitrate on formic hydrogenlyase synthesis. It is already known(2,8) that the presence of nitrate may inhibit formic hydrogenlyase synthesis, and we have found that nitrate inhibits adaptation in the presence of glucose and an amino acid mixture (Table III). Its inhibitory effects may be attributed partly to the formation of nitrite, which also inhibits adaptation(2). On the other hand, as already stated, the presence of nitrate stimulates synthesis of the enzyme in a formate-peptone medium. Probably the energy released by the oxidation of formate by nitrate leads to a stimulation, which more than compensates for the inhibitory effect of nitrate. If opposed effects of this description are involved when nitrate is present, the magnitude of each effect may vary from strain to strain, and this may explain why, in both the strains of *E. coli* with which we have worked, nitrate stimulates adaptation in the presence of excess formate, while in the experiments of Pinsky and Stokes, only inhibitory effects of nitrate are found, even in the presence of excess formate. It is of some importance that the inhibitory effects of ammonium ions on formic hydrogenlyase synthesis may be counteracted by the presence of nitrate (Table III), a fact to be anticipated if nitrate oxidation of formate, or of other substances takes place.

Amino acid requirements for formic hydrogenlyase synthesis. In the presence of glucose and a mixture of the amino acids found in casein hydrolysate, both Strain 1 and Strain 2 show almost identical lag periods and final rates of hydrogen evolution. A marked difference, however, is observed between the 2 strains, in their specific amino acid requirements for formic hydrogenlyase synthesis. The effect on formic hydrogenlyase synthesis of omitting each amino acid from a mixture of the amino acids of a casein hydrolysate has been investigated with the following results. The amino acids, whose omission brings about a significant increase in the lag period and a decrease in final enzymatic activity are, for Strain 1, DL-serine, L-histidine, L-leucine, and DL-aspartate; and for Strain 2, DL-serine, L-histidine, L-arginine, L-leucine, DL-

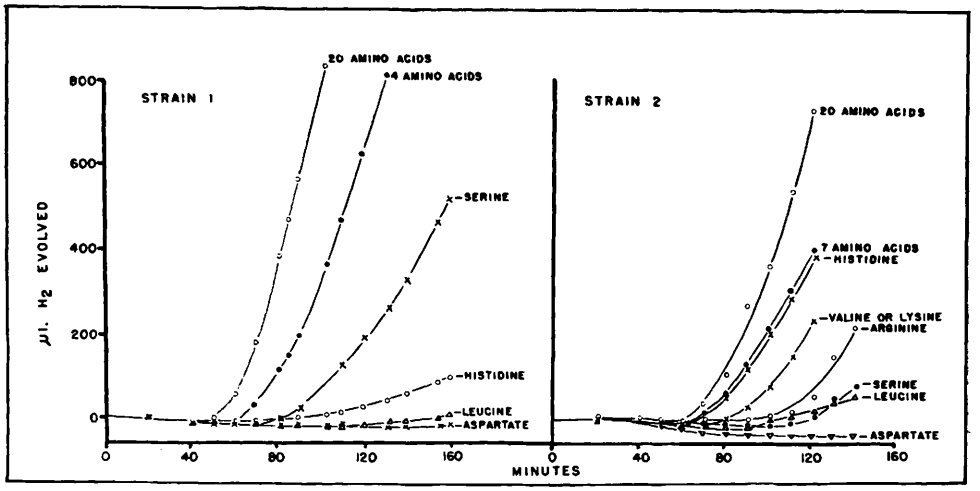

FIG. 1. Effects of amino acids on formic hydrogenlyase synthesis. Glucose (0.033*M*) present in all vessels. Amino acids present in same amount per vessel as in 1 ml of a 5% casein hydrolysate (according to data of Williamson(9)), with addition of 0.75 mg/vessel of DL-norleucine. Where only DL form of the amino acid was available, amino acid was added which gave the appropriate concentration of the L-form. Composition of 4-amino acid mixture in each vessel (for Strain 1) was as follows: DL-serine, 1.67 mg/ml; L-histidine, 0.33 mg/ml; DL-aspartic acid, 1.4 mg/ml; L-leucine, 2.4 mg/ml. Composition of 7-amino acid mixture in each vessel (for Strain 2) was as follows: amino acids as above together with L-arginine, 0.65 mg/ml; L-lysine, 1.0 mg/ml; DL-valine, 1.77 mg/ml.

aspartate, L-lysine, and DL-valine. It is found that for each strain a mixture of amino acids, as specified above, can almost replace a mixture of all the amino acids in a casein hydrolysate as a means of promoting adaptation. Results illustrating this fact and the effect of omission of each amino acid from the simplified mixtures are shown in Fig. 1.

DL-serine, L-leucine, and DL-aspartate are of considerable importance for the enzyme synthesis in both strains. The presence of L-histidine greatly aids the production of the enzyme in Strain 1, but apparently has little effect in Strain 2. L-arginine is of importance for Strain 2, but has no effect on enzyme production in Strain 1. As the results reported in Table II show, none of these amino acids, nor a mixture of 20 present in a casein hydrolysate, has any effect on the formic hydrogenlyase activity of adapted cells. These findings on amino acid requirements agree with those reported by Pinsky and Stokes(2) in showing the importance of DL-serine and L-aspartic acid, and, in one strain, of L-arginine, for formic hydrogenlyase formation. With both our strains of *E. coli* L-glutamate alone can serve as a nitrogen source for formic hydrogenlyase synthesis in the presence of glucose. It seems to be dispensable, however, in the presence of a mixture of amino acids and glucose. When L-glutamate alone is present in a glucose medium, the lag period before the appearance of enzymatic activity is almost twice as long as when an amino acid mixture is used, and the final activity is much lower.

It is possible that variations in amino acid requirements for formic hydrogenlyase formation, between the different strains of *E. coli*, may be due to differences between the rates of amino acid interconversion in these strains.

Summary. 1. A marked stimulation of the synthesis of formic hydrogenlyase by *E. coli* in the presence of sodium formate and peptone is brought about by addition of either DL-aspartate, fumarate, or nitrate under conditions when little or no proliferation takes place. Addition of succinate produces no effect. None of these compounds stimulates activity of the enzyme in pre-adapted cells. Whilst a mixture of glycerol and peptone, or a mixture of fumarate and peptone does not secure enzyme synthesis, a mixture of glycerol, fumarate and peptone is effective. The energy for synthesis in resting cells may thus

be obtained by the interaction of suitable hydrogen donors and hydrogen acceptors. Aspartate is effective presumably because it is converted by aspartase into fumarate. This conclusion is supported by the fact that the presence of ammonium ions inhibits adaptation in a formate-peptone medium, but not in a glucose-*amino acid* medium where energy is secured by glucose breakdown. 2. Certain amino acids are required for the enzyme synthesis. The most effective are leucine, aspartate, histidine, and serine. Requirements for specific amino acids appear to vary with the strain of the organism.

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Toxicity of Non-Ionic Detergents for the Chick Embryo.* (20126)

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A recent investigation by Morton *et al.*(1) showed that the non-ionic detergent Tween 80 in a concentration of 0.5 mg/ml of medium inhibited the growth of fibroblasts in tissue cultures while in adult rats Tween 80 required a concentration of 5-10 g/kg of body weight(2) to be toxic. The higher toxicity of the detergent in tissue cultures raised the question as to whether rapidly growing and less differentiated cells are more sensitive to the detergent than the non-proliferating mature cells. A possibility of comparing the sensitivity to non-ionic detergents of cells in different degrees of proliferative activity and differentiation seemed to be present in the developing chick embryo. Whether non-ionic detergents had a teratogenic effect owing to their surface activity was also of interest because of the great importance of the interaction of cell surfaces during early embryonic development(3). Also, substantial amounts of non-ionic detergents such as Tween 80 are contained in various vitamin preparations which are given to premature infants as well as to mature ones. Therefore, from a clinical point of view, toxicity tests on immature tissue had some interest. Since qualitative and quantitative differences exist in the action of different non-ionic detergents upon different biological systems, such as the tubercle bacillus(4) or the red blood cell(5), it seemed desirable to compare at least two detergents of this type in their effect upon the chick embryo. We chose Tween 80† and Triton X-100† for this comparison.

Materials and methods. Tween 80 is described in the literature as polyoxethylene sorbitan mono-oleate and Triton X-100 as arylalkyl polyether of phenol. The structural formulae are given in the paper by Dubos quoted above(4). Solutions of these substances were made up in distilled water to give the desired amount of material in 0.1 ml. To reach the highest concentrations of Tween 80 the volume was increased to 0.5 ml. The control eggs were injected with 0.6% NaCl solution. All solutions were sterilized by auto-

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