THE EFFECT OF OLEIC ACID AND OF BIOTIN ON THE FORMIC HYDROGENLYASE AND FORMIC DEHYDROGENASE ENZYME SYSTEMS[1]

HERMAN C. LICHSTEIN AND RUTH B. BOYD

Department of Bacteriology and Immunology, University of Minnesota, Minneapolis, Minnesota

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Although oleic acid is recognized as an essential growth factor for several microorganisms (Cohen, Snyder, and Mueller, 1941; Hutner, 1942; Feeney, Mueller, and Miller, 1943; Dubos, 1946; Williams, Broquist, and Snell, 1947; Kitay and Snell, 1950), the basis for the growth promotion by this and other long-chain unsaturated fatty acids is not yet understood. With certain strains of lactic acid bacteria oleic acid is essential only in the absence of biotin, which it apparently replaces in a medium containing sufficient aspartate (Williams, Broquist, and Snell, 1947).

Two theories have developed to explain this biotin-oleate relationship. Williams, Broquist, and Snell (1947) suggested that biotin functions in the synthesis of oleic acid, and when the latter is supplied preformed in the medium, biotin becomes nonessential. Williams and Fieger (1947) and Williams and Williams (1949) suggested that biotin functions as a cell permeability factor and can be replaced by the proper lipides.

In sharp contrast to the growth stimulating effect of unsaturated fatty acids, the toxic action of these substances for various microorganisms has been widely recognized (Kodicek and Worden, 1945; Dubos, 1946; Williams, Broquist, and Snell, 1947) and has led to some difficulty in evaluating the results with these acids.

Some suggestion that the biotin-oleate relationship may not be a direct one may be inferred from the disparity between titration and turbidity assays of biotin-free media containing oleic acid, as reported by Williams and Fieger (1946) and Williams and Andrews (1950) for *Lactobacillus casei*. The latter workers, however, suggested that the oleic effect might be an interference with glycolysis in some way so that only a portion of the normal amount of acid is produced, whereas cell growth is unhampered.

The present paper reports on the effects of oleic acid and of biotin on the formic hydrogenlyase and formic dehydrogenase activity of *Escherichia coli*, and reveals an unexpected property of oleic acid.

MATERIALS AND METHODS

The organism used in a large portion of the studies was a biotinless mutant strain of *Escherichia coli* kindly furnished by Dr. B. D. Davis. In addition, the

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parent wild type was employed as well as other strains of *E. coli* and *Aerobacter aerogenes* from our stock culture collection.

The basal medium employed had the following composition: 0.1 per cent each of KH_2PO_4, K_2HPO_4, HCOOH, and NaCl; 0.07 per cent $MgSO_4 \cdot 7H_2O$; 0.4 per cent $(NH_4)_2SO_4$; 0.05 per cent $C_6H_5O_7Na_3 \cdot 2H_2O$; 0.0001 per cent $Fe_2(SO_4)_3$; and 0.5 per cent acid-hydrolyzed vitamin-free casein (Nutritional Biochemicals Corporation). The final pH was adjusted to 6.5 before autoclaving. The casein was incorporated because of the recent demonstration of the need for certain amino acids for the formation of enzymes concerned with formate metabolism (Billen and Lichstein, 1951).

The biotinless mutant was unable to grow in this basal medium when small washed inocula were employed, unless biotin or oleic acid was supplied. Growth in the presence of oleic acid[2] (10 to 100 μg per ml) without added detoxifying agents was usually about 25 to 35 per cent of that obtained with optimal concentrations of biotin (10^{-3} μg per ml).

The organisms were incubated at 30 C for 15 to 18 hours, the cells harvested by centrifugation, washed once with water, and resuspended in water to give the desired cell concentration.

Conventional manometric techniques were employed in order to follow gas exchange during fermentation. Formic hydrogenlyase was determined by measuring total gas and H_2 production from formate in a Warburg cup at 37 C in an atmosphere of N_2. Paired Warburg vessels were routinely used, one with and one without 20 per cent KOH in the inner well. The former gave a measure of H_2 production, while the latter recorded total gas evolved. Formic dehydrogenase was determined by measuring CO_2 production from formate manometrically at 37 C in an atmosphere of N_2. KOH was not present in the inner well of the cup, and methylene blue (1 ml of 0.01 M) was added to the main compartment. All measurements were made at pH 6 in M/60 phosphate buffer. Formate was tipped in from the side arm after equilibration. When further analyses were desired, the reaction was stopped by the addition of H_2SO_4 to obtain a pH of 2 or less and analytical procedures conducted on an aliquot of the contents of the Warburg cup. Lactic acid was determined colorimetrically by the method of Barker and Summerson (1941), formic acid by the method of Grant (1947), and pyruvate by the method of Friedemann and Haugen (1943). Further details will be presented with the experimental results.

EXPERIMENTAL RESULTS

Studies with the biotinless mutant of E. coli. The first indication that cells grown in the presence of oleic acid differ from those grown with biotin was an unexpected increased rate of fermentation of several substrates by the former cells as compared to the latter (figure 1). Further comparison shows that the the biotin-grown cells exhibit a definite lag period before active fermentation of glucose, pyruvate, or formate begins.

[2] Both the products supplied by Nutritional Biochemicals Corporation and the Hormel Foundation (iodine value 89.28) were employed with similar results.

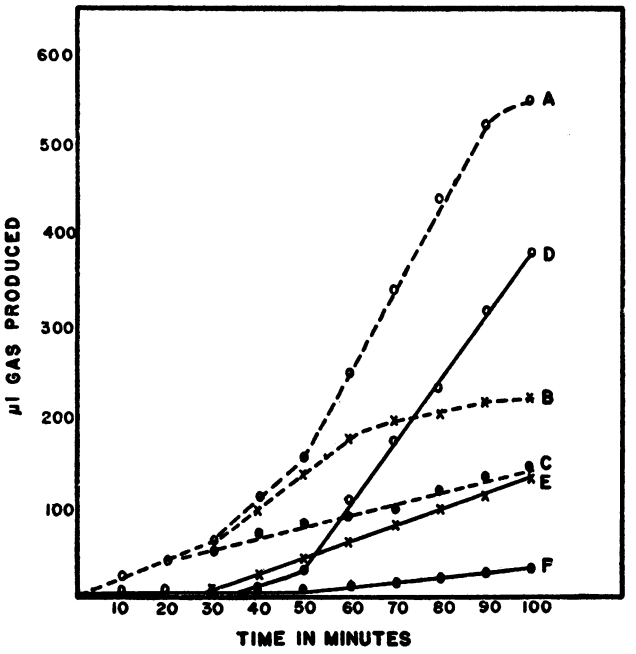

Figure 1. Effect of oleate and biotin on fermentation rate of a biotinless mutant strain of *Escherichia coli*. Curves A, B, C = oleate-grown cells (10 µg/ml) on glucose, pyruvate, and formate, respectively; curves D, E, F = biotin-grown cells (10^{-3} µg/ml) on glucose, pyruvate, and formate, respectively.

TABLE 1

Fermentation of pyruvate and formate by a biotinless mutant of Escherichia coli

	PYRUVATE		FORMATE	
	Biotin-grown cells	Oleate-grown cells	Biotinrgrown cells	Oleate-grown cells
	µl	µl	µl	µl
Total gas	188	260	64	226
Formate	45	0	195	36
Lactate	3	2	0	0
Total gas	151	244	47	248
Formate	85	0	168	83
Lactate	0	0	0	0
Pyruvate	0	0	0	0

Reactions run at 37 C, phosphate buffer pH 6, atmosphere of N_2. Cell concentration 0.18 and 0.24 mg nitrogen per cup. Substrates added in M/10 conc (0.1 ml); final volume 3 ml.

Biotin-grown cells = 10^{-3} µg biotin/ml basal medium.

Oleate-grown cells = 10 µg oleate/ml basal medium.

Inasmuch as manometric data alone are not sufficient for an insight into possible differences in pathway of metabolism, experiments were designed in which lactate, pyruvate, and formate could be determined in addition to total gas evolved. The data (table 1) demonstrate that with this strain of *E. coli* essentially all of the pyruvate is metabolized *via* formate, only a trace going to lactate.

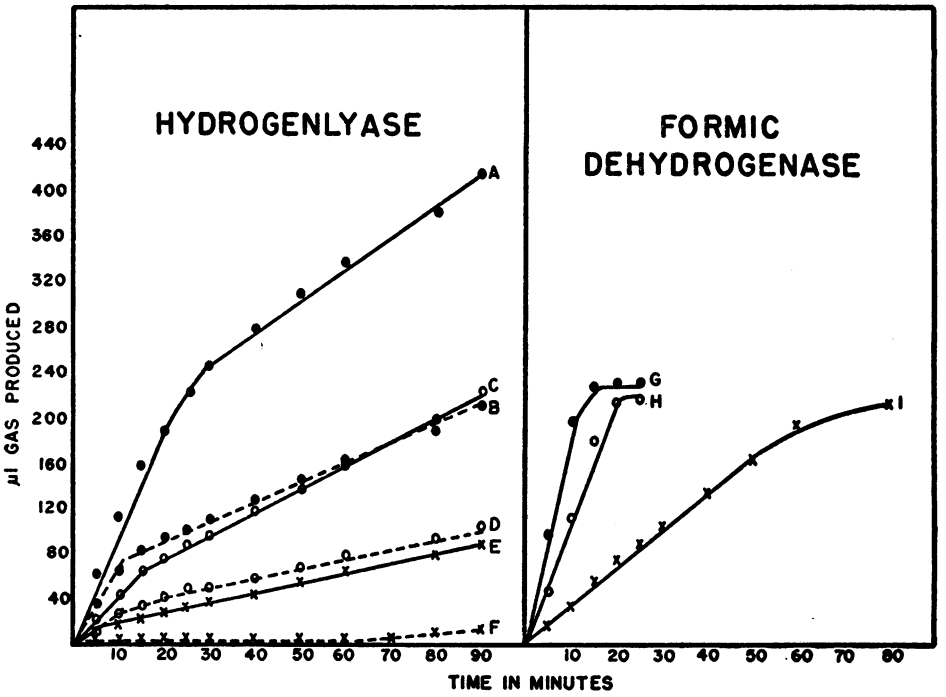

Figure 2. Effect of oleate, biotin, and both agents combined on hydrogenlyase and formic dehydrogenase activity of a biotinless mutant strain of *Escherichia coli*. Biotin concentration = 10^{-3} µg/ml; oleate, 10 µg/ml.

Curve A = total gas production of oleate-grown cells
Curve B = hydrogen production of oleate-grown cells
Curve C = total gas production of biotin- + oleate-grown cells
Curve D = hydrogen production of biotin- + oleate-grown cells
Curve E = total gas production of biotin-grown cells
Curve F = hydrogen production of biotin-grown cells
Curve G = CO_2 production of oleate-grown cells
Curve H = CO_2 production of biotin- + oleate-grown cells
Curve I = CO_2 production of biotin-grown cells

A further indication that the pathway of pyruvate dissimilation is the same for both cells is the fact that no gas is produced from pyruvate or formate by either type of cell when casein is omitted from the medium.

It is quite clear from these data that the cells harvested from a biotin medium exhibit a markedly lowered ability to metabolize formate. This leads quite naturally to an examination of the formic hydrogenlyase and formic dehydrogenase

TABLE 2

Effect of growth conditions on hydrogenlyase and formic dehydrogenase activity of several organisms

ORGANISM	GROWTH CONDITIONS	HYDROGENLYASE ACTIVITY*	FORMIC DEHYDROGENASE ACTIVITY†
E. coli (biotinless mutant)	basal + biotin	0	675
	basal + oleate	410	3,000
	basal + biotin + oleate	110	1,160
E. coli (Davis wild type)	basal	0	710
	basal + biotin	0	410
	basal + oleate	190	1,870
E. coli (biotinless mutant)	basal + biotin	30	280
	basal + oleate	560	1,900
	basal + yeast extract	490	950
E. coli (Davis wild type)	basal	0	160
	basal + oleate	300	700
	basal + yeast extract	410	790
E. coli (Crooks)	basal	240	760
	basal + oleate	410	2,000
	basal	520	3,700
	basal + oleate	1,100	6,000
E. coli (Texas)	basal	420	2,500
	basal + oleate	420	2,600
E. coli (Tennessee)	basal	0	2,700
	basal + oleate	245	3,800
	basal + yeast extract	300	3,700
A. aerogenes (D1)	basal	0	880
	basal + oleate	0	3,200
A. aerogenes (T)	basal	35	350
	basal + oleate	130	550
	basal + yeast extract	200	690

* rate = $\frac{\mu l\ H_2\ \text{produced}}{\text{hr} \times \text{mg nitrogen}}$.

† rate = $\frac{\mu l\ CO_2\ \text{produced}}{\text{hr} \times \text{mg nitrogen}}$.

Conditions as for table 1.

activity of this organism in order to localize the effect more closely. The data presented in figure 2 demonstrate that the biotin-grown cells are deficient in both formic dehydrogenase and formic hydrogenlyase. With the former enzyme

the biotin cells are about one-fifth as active as the oleate cells, while it is doubtful whether the biotin cells had any hydrogenlyase activity at all, since essentially no H_2 was produced during the course of the experiment. The total gas production of the biotin-grown cells may then be a reflection of the ability of the cells to carry out the formic dehydrogenase system to a limited extent without added hydrogen acceptors. The fact that such acceptors are present in the washed cell suspensions was demonstrated by the rate of H_2 uptake, which, although low,

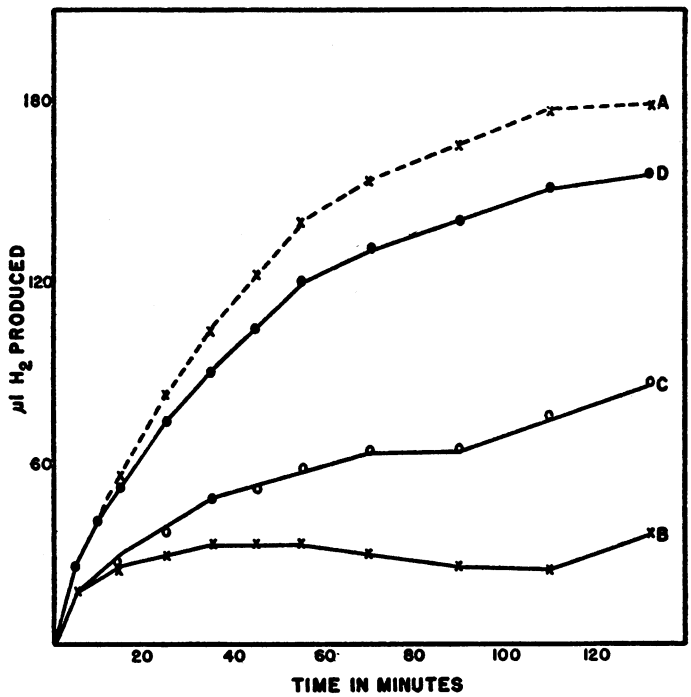

Figure 3. Effect of oleate and biotin on the formic hydrogenlyase activity of resting cell suspensions of *Escherichia coli* (biotinless mutant).

Curve A = cells grown in oleate (100 µg/ml)
Curve B = cells grown in biotin (10^{-4} µg/ml)
Curve C = cells grown in biotin (10^{-4} µg/ml) (10^{-1} µg biotin added at zero time)
Curve D = cells grown in biotin (10^{-4} µg/ml) (100 µg oleic acid added at zero time)

was very definitely present. It is of further interest that the cells grown in the presence of both oleate and biotin occupy an intermediate level of activity in both systems. This has been observed consistently and suggests that biotin, even in the presence of adequate oleate, in some manner antagonizes the effect of the fatty acid.

Studies with other strains. On the basis of the results with the biotinless mutant strain of *E. coli*, the question that arose quite naturally was whether the need for oleic acid had any relationship to the biotin requirement imposed on this organism by radiation.

The wild type of *E. coli* from which this mutant had been obtained was tested, and the results (table 2) were comparable to those obtained with the mutant, proving that the requirement for oleate in the production of these enzymes is not caused by the radiation. It is of interest to note that the wild type of *E. coli* is less active than the mutant strain even when grown in oleate or yeast extract. We interpret these findings to the ability of the wild type to synthesize biotin so that the cells are growing in both biotin and oleate when the fatty acid is added to the medium. Comparing this with the mutant strain grown in a similar fashion, it may be noted that the activity is reduced when biotin is present.

Studies with other strains show that, while they all produce a formic dehydrogenase system which is substantially increased when grown in the presence of oleate, the ability to produce the hydrogenlyase enzyme is restricted. The negative results given are based on the absence of H_2, although in most instances some CO_2 was produced, suggesting once more that a low formic dehydrogenase activity may be conducted in the absence of added H_2 acceptors.

Stimulation experiments. In an attempt to shed more light on the role of oleate and biotin, it was deemed advisable to study the effect of these substances on the formic hydrogenlyase activity of washed cell suspensions. The biotinless mutant strain of *E. coli* was employed, and additions of either biotin or oleate were made from the side arm of the Warburg cup simultaneously with the addition of formate. The results of one such experiment are graphically presented in figure 3.

It may be seen that cells harvested from the basal medium containing 10^{-4} μg of biotin per ml exhibit essentially no activity until 110 minutes, when hydrogen evolution is noted. This has been consistently observed, although this lag period has varied between 55 and 140 minutes. It is noted further that the cells harvested from the basal medium containing 100 μg of oleic acid per ml have an active formic hydrogenlyase. Although the concentration of oleate necessary during growth for hydrogenlyase activity has not been established, 10 μg per ml give essentially the same results. Of particular importance is the profound and immediate stimulation of activity in the biotin-grown cells upon the addition of 100 μg of oleic acid. In most experiments the addition of 10 μg of this fatty acid is almost as effective, while 1 μg is inactive.

It would appear from these data that the biotin-grown cells contain the hydrogenlyase apoenzyme and that after a suitable incubation time a cofactor is synthesized, at least in small amounts. The addition of oleic acid apparently results in immediate cofactor synthesis by the biotin-grown cells, suggesting that oleate or some substance derived from this fatty acid functions in some manner as a cofactor for formic hydrogenlyase.

The addition of biotin to the cells grown in the presence of 10^{-4} μg of this vitamin per ml results in some stimulation of activity, but the character of the stimulation differs from that obtained with oleate in several respects. First, the magnitude of stimulation is never very great, and secondly, the rate often levels off like that observed with the biotin cells (curve B) and then proceeds again after a period of time. It is pertinent to these data that cells harvested

from media containing higher concentrations of biotin (10^{-3} μg to 10^{-1} μg per ml) show little or no ability to be stimulated by either oleate or biotin in the concentrations tested.

DISCUSSION

The results of these studies provide evidence that oleic acid is involved in the formic hydrogenlyase and formic dehydrogenase enzyme systems. The former enzyme appears more dependent on the presence of this fatty acid than does the latter.

Although the precise mechanism and specificity of action of oleic acid are still obscure, the experimental results with the hydrogenlyase enzyme employing resting cell suspensions suggest that this fatty acid or some substance readily derived from it functions as a cofactor of this enzyme system.

The role of biotin is not clear. When incorporated into the growth medium, optimal or excess amounts of this vitamin uniformly decrease the activity of both enzymes, and furthermore may even prevent the oleic stimulation of resting cells which is observed when low biotin cells are employed (10^{-4} μg per ml). On the other hand, biotin exhibits some stimulatory effect on the formic hydrogenlyase activity of the resting cells (10^{-4} μg biotin per ml). It may be that although the biotin-oleate relationship is still obscure, clarification may proceed more rapidly by a study of these enzyme systems rather than by growth replacement studies alone.

SUMMARY

Data are presented demonstrating that oleic acid is required for optimal activity of both the formic hydrogenlyase and formic dehydrogenase enzyme systems of *Escherichia coli*.

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