STUDIES ON THE FORMIC HYDROGENLYASE SYSTEM OF BACTERIA

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Several laboratories have reported recently on the nature and cofactor requirements of the formic hydrogenlyase system of bacteria (Lichstein and Boyd, 1953a,b; Gest, 1952; Gest et al., 1953; Broquist and Kohler, 1953; Lascelles, 1948; Grunberg-Manago et al., 1951; Sevag et al., 1945). Although the net result of these reports has been to renew interest and to help measurably in an elucidation of this enzyme system, there remain several areas of disagreement.

The present paper is concerned with the nature of the carbohydrate stimulation of the formic hydrogenlyase system reported by Lascelles (1948) and Broquist and Kohler (1953), and the problem of whether hydrogen production is dependent on a single enzyme system (formic hydrogenlyase) or a family of enzymes (formic dehydrogenase and hydrogenase).

Data are presented herein which prove that the yeast extract factor(s) reported by Lichstein and Boyd (1952) is not identical with the carbohydrate effect of Lascelles (1948) or of Broquist and Kohler (1953), and that the formic hydrogenlyase system is a separate entity not obligatorily dependent on either formic dehydrogenase or hydrogenase activity. In addition, certain interesting variations are presented among different species of bacteria.

MATERIALS AND METHODS

The organisms studied were *Aerobacter aerogenes* (strain D-1), the Crookes, Texas, and biotinless mutant strains of *Escherichia coli*, and *Proteus vulgaris*. The composition of the basal synthetic medium, the method of preparing resting cell suspensions and dried cells are given in a previous communication (Lichstein and Boyd, 1952), whereas the methods of enzyme assay are cited by Lichstein and Boyd (1953a). Unless otherwise specified *A. aerogenes* was grown in the basal medium to which 0.1 per cent glucose was added aseptically after sterilization, and the biotinless mutant strain of *E. coli* was cultured in the basal medium to which were added \(10^{-4}\) \(\mu\)g of biotin per ml before autoclaving. Deep stationary cultures at 30 C for 16 to 18 hr were employed routinely.

Reducing sugar was determined quantitatively by a modification of the method of Folin and Malmros (Umbreit et al., 1949).

The hydrolyzed yeast extract (HYE) was Difco yeast extract autoclaved at 121 C for 3 hr in 0.9 N \(\text{H}_2\text{SO}_4\) followed by neutralization with \(\text{Ba(OH)}_2\).

EXPERIMENTAL RESULTS

The effect of glucose and hydrolyzed yeast extract. Lascelles (1948) and more recently Broquist and Kohler (1953) have reported that glucose and certain other fermentable sugars exert a marked stimulatory effect on the formic hydrogenlyase system. The former worker emphasized that glucose did not alter appreciably the final rate of hydrogen evolution from formate but was effective in reducing or even eliminating the lag often observed in the initial rates of hydrogen production. She concluded that glucose fermentation provides a factor essential for formic hydrogenlyase activity. Broquist and Kohler (1953), however, concluded that the stimulation of the formic hydrogenlyase system by extracts of yeast as reported by Lichstein and Boyd (1952) is due to the presence of reducing sugar. The experiments presented in this section were designed to study the nature of the carbohydrate stimulation of the formic hydrogenlyase system and to compare this with the stimulations produced by hydrolyzed yeast extract.

The data given in figures 1 and 2 reveal

1 Kindly furnished by Dr. B. D. Davis.
several points of interest concerning the stimulatory effect of glucose and of hydrolyzed yeast extract on the formic hydrogenlyase enzyme system. Fresh cellular suspensions of *Aerobacter aerogenes* were stimulated by hydrolyzed yeast extract without a detectable lag period, suggesting the presence of preformed cofactor, while the stimulation resulting from the addition of glucose required a definite period of time (figure 1). It is seen further that the lag period before stimulation by glucose was lengthened as the cell suspensions were aged in the refrigerator. With *E. coli*, strain Texas, the lag period before stimulation by added glucose was much longer than with *Aerobacter aerogenes*, and after aging of the cell suspensions no stimulation by glucose was noted, while hydrolyzed yeast extract increased the rate of formic hydrogenlyase very markedly. Determination of reducing sugar revealed that all of the added glucose had been metabolized by the cells. The results presented in figure 2 demonstrate that semicarbazide, while reducing only slightly the stimulatory effect of hydrolyzed yeast extract, almost completely abolished the ability of glucose to stimulate the formic hydrogenlyase system, presumably because it inhibits the breakdown of glucose beyond pyruvate.

These results demonstrate that glucose must be metabolized in order to exert a stimulatory effect on the formic hydrogenlyase system and that the effect of glucose reported here differs from that described by Lascelles (1948) in that the effect is not primarily on the lag period. The data also suggest that the factor or factors present in hydrolyzed yeast extract which stimulate the formic hydrogenlyase system are not reducing sugars as claimed by Broquist and Kohler (1953). The differences observed between the effect of glucose on the formic hydrogenlyase system of *Aerobacter aerogenes* and *E. coli*, strain Texas, suggest further that the carbohydrate must be metabolized in a distinct manner since in the case of aged suspensions of the latter organism glucose was found to disappear without any demonstrable effect on the formic hydrogenlyase activity of the cells.

Attempts to determine if known metabolic products of glucose fermentation were capable of stimulating formic hydrogenlyase activity in *Aerobacter aerogenes* revealed that pyruvate also was active. Lactate, glycerol, acetate, ethanol, succinate, *a*-ketoglutarate, citrate, acetylmethylcarbinol, diacetyl, propionate, and ribose were not effective in stimulating the formic hydrogenlyase system of this organism when tested in concentrations of 2 μM.

*Nondependence of formic hydrogenlyase stimulating activity of hydrolyzed yeast extract on its reducing sugar content*. The experimental results already presented suggest that the stimulatory activity of hydrolyzed yeast extract is not dependent on the reducing sugar content. However, inasmuch as Broquist and Kohler (1953) claimed that the activity of yeast in this system was due entirely to its content of reducing sugars, it became mandatory to develop additional experiments to clarify this controversy.

The first such experiment consisted of a titration of glucose and of hydrolyzed yeast extract with respect to their formic hydrogenlyase stimulating activity and a comparison of activity
Figure 2. Effect of semicarbazide on the stimulatory action of glucose and hydrolyzed yeast extract on the formic hydrogenlyase system.

Bacterial cell suspensions refrigerated 4 days; semicarbazide (SC) conc = 0.02 μM; glucose conc = 2 μM; hydrolyzed yeast extract (HYE) conc = 2 mg; other conditions as for figure 1.

Based on the reducing sugar content of the yeast extract. In order to obviate the need for endogenous controls to determine the amount of hydrogen liberated from glucose or yeast extract, these stimulants were preincubated with the cells in the absence of formate and the latter added to the main compartment of the Warburg vessel only after all endogenous rates had fallen to zero. A comparison of the relative stimulatory effects of the hydrolyzed yeast extract and glucose (figure 3) based on the reducing sugar content of the former (100 μg per mg hydrolyzed yeast extract) revealed that the yeast was more active than could be accounted for by its reducing sugar content. Such results offer additional evidence contradictory to the claims of Broquist and Kohler (1953). Thus, for example, 360 μg (2 μM) of glucose stimulated formic hydrogenlyase activity while 108 μg (0.8 μM) were inactive. In contrast, 0.06 mg hydrolyzed yeast extract ("glucose equivalent" = 5 μg) exhibited slight activity while 0.1 mg ("glucose equivalent" = 10 μg) possessed a definite ability to stimulate the formic hydrogenlyase system. Another point of interest was the rate of hydrogen production (QH₂ = μL H₂ produced per hr per mg cell N) before and after preincubation; no addition cells were 900 and 700, respectively, before and after preincubation, the rates increased to 2,600 and 2,650, respectively, in the presence of two mg hydrolyzed yeast extract, whereas with two μM glucose the rates were 3,400 and 1,400, respectively. Thus, the rate of formic hydrogenlyase activity differed markedly before and after preincubation with glucose, suggesting that the stimulatory action of glucose may involve more than one mechanism. The preincubation results appear routinely to provide consistent data and to support the hypothesis that the fermentation of glucose under certain conditions yields a substance necessary for the optimal activity of the formic hydrogenlyase enzyme system.

In contrast to the earlier work of Lascelles (1948) where the effect of glucose appeared to be primarily in decreasing or eliminating the lag period before hydrogen production from formate was noted, the results herein described show...
marked differences in the over-all rate of the reaction. Studies with other strains of bacteria revealed that these differences may be a problem of strain variation. Experiments with E. coli (biotinless mutant) and P. vulgaris gave results essentially identical with A. aerogenes as far as the character of the stimulation by glucose is concerned. However, the results with E. coli, strain Crookes, were in general agreement with Lascelles (1948) since glucose affected primarily the initial rate of hydrogen production from formate. These results emphasize the strain variations that occur repeatedly in studies of the physiology of bacteria and which must be considered before generalizations can be made.

In order to obtain more direct evidence that the fermentation of glucose yields a substance which is required as a cofactor for the formic hydrogenlyase system, the following experiment was designed. Suspensions of A. aerogenes were incubated in Warburg vessels at 37 C under nitrogen in phosphate buffer, pH 6. No further additions were made to one series of cups, a second series contained glucose, and the third series was incubated with hydrolyzed yeast extract. After a period of incubation sufficient to insure removal of all added glucose, the bacterial cells were separated by centrifugation, resuspended in water, and boiled for 10 minutes. These "kochschafts" were tested for formic hydrogenlyase stimulating activity on A. aerogenes cell suspensions of three different nutritional backgrounds: (1) basal grown, (2) grown in basal medium plus two per cent glucose, and (3) grown in basal medium plus one per cent hydrolyzed yeast extract. The results presented (table 1) demonstrate that "kochschafts" of bacterial cells previously incubated with glucose or hydrolyzed yeast extract exhibited definite activity with respect to the formic hydrogenlyase system whereas the control "kochschaft" was inactive. It should be stated that this is to some extent a concentration effect since larger amounts of basal "kochschaft" did stimulate the system albeit to a decidedly smaller extent than the "kochschafts" prepared from the glucose or yeast extract exposed cells. These data support those already presented to demonstrate that the
fermentation of glucose under certain conditions
leads to the formation of a product which appears
to function as a cofactor for the formic hydrogenlyase
system.

It is important to note (table 1) that the cells
which were harvested from the basal medium
supplemented with glucose were no longer
stimulated by the addition of this sugar to the
resting cell suspensions but were stimulated
markedly by hydrolyzed yeast extract or "kochsafs" prepared from bacterial cells previously
exposed to hydrolyzed yeast. Such results
afford convincing evidence contesting the claims
of Broquist and Kohler (1953).

Previous reports from this laboratory (Lich-
stein and Boyd, 1952, 1953a) have suggested
that hydrolyzed yeast extract contains perhaps
two factors which are required for optimal ac-
tivity of the formic hydrogenlyase system. The
data of table 2 offer additional evidence for the
existence of two distinct factors in hydrolyzed
extracts of yeast and suggest that the increased
activity after hydrolysis is not due to the release
of a fermentable carbohydrate since semicar-
baside did not eliminate its activity. Further,
although the activity of two mg of hydrolyzed
yeast extract in the presence of semicarbazide
was not greater than an equal amount of un-
hydrolyzed yeast, the activity of 5 mg of the
hydrolyzed yeast when tested with semicarbazide
was markedly more active than 5 mg of the un-
hydrolyzed material. It is, however, possible that
the factor formed as a result of the fermentation

| TABLE 1 |
| Effect of "kochsafs" on the formic hydrogenlyase activity of Aerobacter aerogenes |

<table>
<thead>
<tr>
<th>ADDITIONS</th>
<th>FHL ACTIVITY*</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Basal cells</td>
</tr>
<tr>
<td>None</td>
<td>650</td>
</tr>
<tr>
<td>Glucose, 2 µM</td>
<td>1,100</td>
</tr>
<tr>
<td>HYE, 2 mg.</td>
<td>1,400</td>
</tr>
<tr>
<td>Basal kochsafs</td>
<td>700</td>
</tr>
<tr>
<td>Glucose kochsafs</td>
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<tr>
<td>HYE kochsafs</td>
<td>1,200</td>
</tr>
</tbody>
</table>

* FHL activity = µL H₂ produced/hr/mg cell N.

Conditions as for figure 1; kochsafs prepared
as described in text and tested at a conc of 0.04
mg cell N.

Aerobacter aerogenes, 0.07 mg N per cup, sus-
pension 2 days in refrigerator. Preincubated
with or without additions for 180 min. Semi-
carbazide conc = 0.02 µM; HYE = hydrolyzed
yeast extract; YE = yeast extract; other condi-
tions as for figure 1.

of glucose or pyruvate is present preformed in
extracts of yeast.

Identity of the formic hydrogenlyase system.
The polemics concerning the nature of the formic
hydrogenlyase enzyme system (Umbreit, 1951)
appears to be resolved partially in view of the
recent reports of Grunberg-Manago et al. (1951)
and Lichstein and Boyd (1953b). The former
workers demonstrated hydrogen production
from formate in the presence of sodium hypophos-
phite which inhibited formic dehydrogenase
activity, while the latter group reported hy-
drogen production in the absence of an active
dehydrogenase.

The experiments of Grunberg-Manago et al.
(1951) were repeated in this laboratory with
results (table 3) that are in agreement with
theirs. It appears that the formic dehydrogenase
system can be eliminated by treatment of the
cells with NaH₂PO₂ so long as sufficient time of
exposure is allowed. Under the same conditions
hydrogen production from formate (formic
hydrogenlyase system) was reduced only slightly.
It should be emphasized that NaH₂PO₂ was
found to exert a marked inhibitory action on
The effect of sodium hypophosphite on the formic hydrogenlyase and formic dehydrogenase activity of several strains of bacteria

<table>
<thead>
<tr>
<th>ORGANISM</th>
<th>TIME IN MIN</th>
<th>FHL ACTIVITY*</th>
<th>FD ACTIVITY†</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>NaH₂PO₄</td>
<td>Control</td>
</tr>
<tr>
<td>Aerobacter aerogenes</td>
<td>0-10/3,100</td>
<td>2,900/3,900</td>
<td>1,000/1,100</td>
</tr>
<tr>
<td></td>
<td>10-20/1,900</td>
<td>1,400/2,700</td>
<td>0</td>
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<td></td>
<td>30-50/1,400</td>
<td>1,100/1,100</td>
<td>0</td>
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<tr>
<td>Escherichia coli, strain Texas</td>
<td>30-50</td>
<td>800/1,100</td>
<td>0</td>
</tr>
<tr>
<td>E. coli, strain Crookes</td>
<td>30-50</td>
<td>1,060/1,500</td>
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<tr>
<td>E. coli, strain biotinless</td>
<td>30-50</td>
<td>1,350/1,800</td>
<td>0</td>
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</tbody>
</table>

* FHL activity = \( \mu \text{L} \) \( \text{H}_2 \) produced/hr/mg cell N.
† FD activity = \( \mu \text{L} \) \( \text{CO}_2 \) produced/hr/mg cell N.
NaH₂PO₄ conc = 0.002 M.

The formic hydrogenlyase system if the cells were incubated for some time with this substance prior to the addition of formate. The hydrogenase enzyme was not affected by NaH₂PO₄ which is in agreement with the results of Grunberg-Manago et al. (1951).

During the course of these studies it was found that the activity of formic hydrogenlyase, formic dehydrogenase, and hydrogenase varied markedly with respect to the age of the washed cell suspensions and the nature of the growth medium. It was decided therefore to investigate this aspect more carefully in the hope that one might find conditions which would yield bacterial suspensions possessing formic hydrogenlyase activity without one or both of the other enzymes. The results presented (table 4) reveal that the concentration of glucose in the growth medium played an important role in the stability of these enzymes to refrigeration of the resting cell suspensions. In this particular experiment the formic hydrogenlyase system was increased initially in direct proportion to the concentration of glucose in the growth medium, and the stability of the system to aging correlated in a similar fashion. The formic dehydrogenase enzyme was not affected in initial activity by the presence or absence of glucose during growth, but the stability of the system varied inversely with the concentration of glucose in the medium. This effect is similar to that observed in studies with aspartic acid deaminase (Boyd and Lichstein, 1952) where certain variations in nutrition, while not affecting the initial activity of the enzyme, influenced markedly the stability of the system to successive washings. The hydrogenase system (table 4) appeared to be quite dependent on the presence of glucose in the growth medium. From the results presented it is evident that hydrogen production from formate can occur in the absence of an active hydrogenase and/or formic dehydrogenase system. Although the time of aging of the cell suspensions required to demonstrate this phenomenon has been variable, these results have been repeated often in this laboratory, and when considered in conjunction with the other data reported in this and prior papers, they support the identity of a formic hydrogenlyase system.

**SUMMARY**

Data are presented which demonstrate that the fermentation of glucose under certain conditions results in the formation of a substance...
which is required for the optimal activity of the formic hydrogenlyase enzyme system. Certain interesting and important strain variations are noted with respect to the effect of glucose on this system.

Abundant evidence is given to show that the stimulatory action of hydrolyzed yeast extract on the formic hydrogenlyase system cannot be accounted for by its content of reducing sugar.

Results of studies with inhibitors as well as with bacteria grown under different nutritional conditions yield convincing evidence that formic hydrogenlyase is a distinct enzyme system and is not dependent on either formic dehydrogenase or hydrogenase activity.

REFERENCES


