ENZYMATIC ASPECTS OF GAS FORMATION BY SALMONELLA

J. L. STOKES

Western Utilization Research Branch, Agricultural Research Service, United States Department of Agriculture, Albany 10, California

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Bacteria of the genera *Escherichia*, *Aerobacter*, *Proteus*, and *Salmonella* usually ferment sugars with the formation of both acid and gas. The gas is a mixture of hydrogen and carbon dioxide and arises from the decomposition of formic acid produced as an intermediate in the metabolism of the carbohydrates (Pakes and Jollyman, 1901; Harden, 1901):

$$\text{Sugar} \rightarrow \text{HCOOH} \rightarrow \text{H}_2 + \text{CO}_2$$

Harden showed that the characteristic lack of gas formation by *Salmonella typhosa* is due to the inability of the organisms to split formic acid and the latter therefore accumulates in the culture medium. This is true also for non-gas-forming or anaerogenic strains of *Salmonella paratyphi* strain B (Tasman and Pot, 1934) and of anaerogenic coliform bacteria (Ordal and Halvorson, 1939; Gordon and Stickland, 1949).

The enzyme or enzyme system responsible for the splitting of formic acid was named formic hydrogenlyase by Stephenson and Stickland (1932). Their investigations and the subsequent work of others (Ordal and Halvorson, 1939; Gest and Peck, 1955) have supplied much evidence, mostly circumstantial, that the decomposition of formic acid into H_2 and CO_2 is a relatively complex process and may require the participation of two enzymes, hydrogenase and formic dehydrogenase, and also intermediate electron carriers. This aspect of the splitting of formic acid is not yet fully clear. Stephenson and Stickland (1933) found that formic hydrogenlyase is an adaptive enzyme. It is not synthesized when *Escherichia coli*, for example, is grown under aerobic conditions in the absence of sugar, e. g. on nutrient agar plates (Stokes, 1949) but will appear when suspensions of such cells are incubated for about an hour with formate and certain nutritional supplements. The latter include a source of energy such as glucose and several amino acids presumably required for the synthesis of the enzyme (Pinsky and Stokes, 1952*a*). Interestingly, adaptation proceeds better in old cells than in young ones (Pinsky and Stokes, 1952*b*).

Most studies of formic hydrogenlyase have been made with coliform bacteria. The present investigations were undertaken to determine some of the properties of hydrogenlyase in *Salmonella* and the relation of this enzyme and the possibly associated enzymes, hydrogenase and formic dehydrogenase, to gas formation in normal and anaerogenic strains.

MATERIALS AND METHODS

Nine cultures of the genus *Salmonella* were used. These included four strains of typical gas-forming cultures, *S. oranienburg* strain 200E, *S. pullorum* strain 3083, *S. typhimurium* strain TM-1, and *S. senftenberg* strain 775W and five non-gas forming or anaerogenic strains, *S. gallinarum* strain 222, *S. typhimurium* strain 1494, *S. montevideo* strain 3A, *S. paratyphi* B strain 12A, and *S. oranienburg* strain 3861.

The gas-forming strains were grown on trypticase soy agar plates in order to obtain cells devoid of hydrogenlyase for adaptation and other experiments. Cells containing hydrogenlyase were obtained by growing the cultures in a broth consisting of 1 per cent each of peptone, yeast extract, and glucose dissolved in 0.02 M phosphate buffer, pH 7.0. The broth was distributed in 150 ml quantities in 250-ml Erlenmeyer flasks to provide relatively deep layers suitable for fermentation and therefore hydrogenlyase formation. The anaerogenic strains were grown only in the glucose broth. The cells were harvested after growth for one day at 35 C and suspended, without washing, in sufficient 0.1 M phosphate buffer, pH 7.2, to yield a turbidity of 450 in the Klett-Summerson colorimeter (660 mμ filter). Such suspensions contain approximately 2 mg of cells, dry weight, per ml. For the experiments on the fermentation of glucose, the cells were suspended in 0.0075 M $NaHCO_3$.

Conventional manometric methods were used in measuring the enzymatic activities of the cell suspensions. Each vessel received 2 ml of cell suspension. One- or two-tenths ml of 0.1 M or 0.05 M substrate, i.e., 5 or 10 micromoles, were placed in the side cup. Ten per cent KOH was used in the center well to absorb CO_2. The gases, N_2, H_2, and N_2 containing 5 per cent CO_2 were freed of oxygen by passage over heated copper gauze. The bath temperature was 30 C.

Hydrogenlyase was measured as H_2 evolution by the cells from 10 μmoles sodium formate in an N_2 atmosphere and in the presence of KOH. Hydrogenlyase-deficient cells were adapted to form the enzyme by tipping into the suspensions 10 μmoles of glucose and 0.2 ml of 10 per cent casein hydrolyzate in addition to the formate. Hydrogenase was measured as H_2 consumption by the cells in the presence of 10 μmoles sodium fumarate or 10 μmoles methylene blue in an H_2 atmosphere with KOH in the center well. Formic dehydrogenase activity was followed by O_2 consumption in an atmosphere of air and in the presence of 10 μmoles of formate and KOH. The rate and extent of fermentation of glucose was determined by the evolution of CO_2 due to the interaction of the fermentation acids with the bicarbonate of the cell suspensions, and the total amount of acid formed was calculated from the difference between initial and residual bicarbonate. The latter were determined by tipping in 0.2 ml of 2N H_2SO_4.

To detect formic acid, cultures were steam distilled at pH 2 and analyses were made on the volatile acid fraction by the $HgCl_2$ method (Association of Official Agricultural Chemists, 1945).

RESULTS

Gas-producing strains. Cells from broth. Hydrogenlyase. Salmonella cultures which normally produce gas during the fermentation of sugars exhibit a consistent enzymatic pattern. When such strains are grown in deep layers of glucose broth and the resulting cells are harvested and placed in contact with glucose or formate, they bring about an immediate and rapid evolution of hydrogen. The kinetic aspects of the decomposition of formate by *S. oranienburg* which are typical are shown in figure 1. The liberation of H_2 continues until the theoretically maximum amount from 10 μmoles of formate, 224 μL, is obtained and the process is completed in about 1½ hr. The cells contain therefore, an active formic hydrogenlyase.

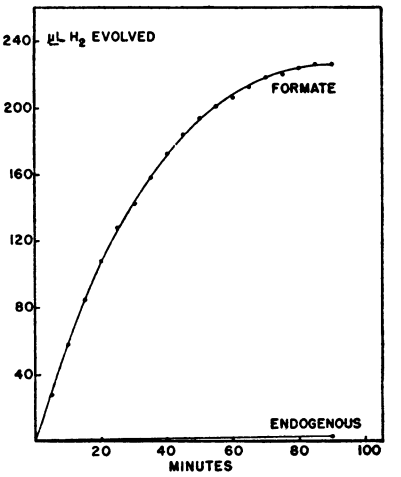

Figure 1. Hydrogenlyase activity of *Salmonella oranienburg* cells from glucose broth medium.

The activity of the hydrogenlyase in Salmonella is greatly influenced by the imposed pH level. This is shown for *S. typhimurium* in figure 2. The cells were grown in glucose broth and suspended in 0.1 M phosphate buffer at each of the indicated pH values. The optimum pH for hydrogenlyase is 5.1, the lowest level tested, and the rate of H_2 evolution decreases with increase in pH of the phosphate buffer. For *S. oranienburg* the optimum was also in the range of pH 5.1 to 5.5.

Stephenson and Stickland (1932) reported that the optimum pH for hydrogenlyase activity of *Escherichia coli* is 7.0 and that activity decreases rapidly at lower and higher pH levels. Thus, the activity at pH 6.0 and pH 8.0 was only about 20 per cent of that obtained at pH 7.0. In a later publication, however, Stephenson (1937) stated that the optimum pH varied with the formate concentration and shifted towards the acid side as the amount of formate was decreased. With 0.005 M formate as final concentration, which is that used in our experiments, the optimum was pH 6.3. It would appear, therefore, that the hydrogenlyase of Salmonella is favored more by acid reactions than that of *E. coli*. But this difference may be more apparent than real. The results of some of our earlier experiments with *E. coli* closely parallel the present data with Salmonella. Q_{H_2} values, μL H_2

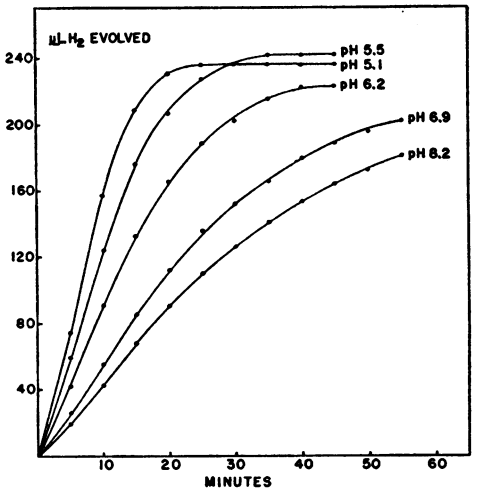

Figure 2. Effect of pH on the hydrogenlyase activity of *Salmonella typhimurium*.

evolved/mg dry cells/hr, obtained with *E. coli* were 101, 88, 67, 60, 33, and 18 for corresponding pH levels of 5.3, 6.0, 6.5, 7.0, 7.5 and 8.1.

Hydrogenase. The Salmonella cells harvested from the glucose broth cultures, and which contain hydrogenlyase, also activate molecular hydrogen. With fumarate as acceptor the following reaction occurs:

$$H_2 + \text{fumarate} \rightarrow \text{succinate}.$$

The rate and extent of H_2 consumption with *S. oranienburg* in the presence of fumarate is shown in figure 3. The cells rapidly take up H_2. There is a small amount of H_2 consumption due to the endogenous metabolism of the cells. When methylene blue is substituted for fumarate, the rate of H_2 consumption usually increases greatly. This difference is much more pronounced in the case of aerobically grown cells, as will be described later, and is probably a reflection of the rate-limiting effect of fumarate activation or of the intermediary electron carriers involved in the reaction.

Formic dehydrogenase. The above-described Salmonella cells also catalyze a rapid uptake of O_2 in the presence of formate. This would seem to indicate that the cells contain formic dehydrogenase, the enzyme involved in the reaction:

$$HCOOH + \tfrac{1}{2} O_2 \rightarrow H_2O + CO_2.$$

But as has been often emphasized by Gest (1954) and others, the significance of this reaction in cells which contain both hydrogenlyase and hydrogenase is not clear since O_2 uptake could also be due to the oxidation of H_2 released from the formate by hydrogenlyase and catalyzed by hydrogenase. Our earlier experiments with *E. coli* have suggested that this difficulty can be eliminated by carrying out the formate oxidation in borate buffer at pH 9.0, since under these conditions the hydrogenlyase reaction is virtually completely suppressed. This has not been tested, as yet, with Salmonella. Gest and his associates (Gest 1954), however, have been able to repress hydrogenlyase activity in cell-free extracts of *E. coli* without inhibiting formic dehydrogenase by adjusting the extracts to pH 7.2.

Cells from agar. When the gas-producing strains of Salmonella are grown on agar plates of glucose-free media, the harvested cells, in contrast to those from glucose broth, cannot release gas from either glucose or formate. This inactivity of the cells is not due to any inability to metabolize glucose. Actually, they ferment glucose rapidly. The course of a typical fermentation by *S. pullorum* suspended in bicarbonate buffer is shown in figure 4. The 5 μmoles of glucose were completely fermented in 35 min. The CO_2 balances for this particular fermentation and for those of other Salmonella (table 1) also indicate that the cells from agar ferment glucose without the formation of gas. The close agreement between the initial HCO_3^--CO_2 and the sum of the CO_2 liberated by the fermentation acids and the residual HCO_3^--CO_2 proves that only acid was formed. If CO_2 also had been produced metaboli-

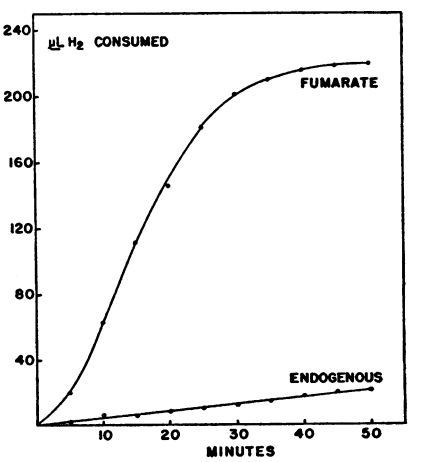

Figure 3. Hydrogen consumption by *Salmonella oranienburg*.

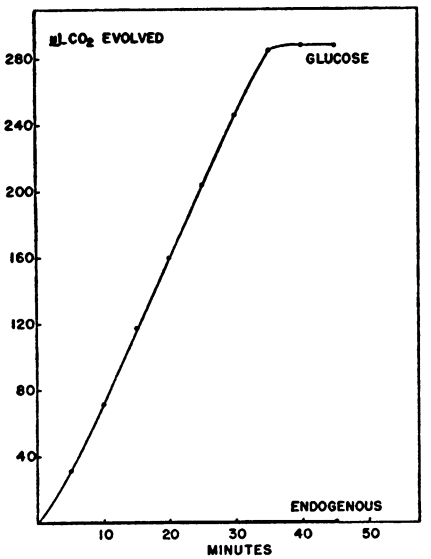

Figure 4. Fermentation of glucose by *Salmonella pullorum.*

TABLE 1

Carbon dioxide balances in the fermentation of 5 μmoles of glucose by Salmonella

	S. pullorum	*S. oranienburg*	*S. typhimurium*
	μl	μl	μl
Initial HCO_3^--CO_2	580	464	519
CO_2 liberated	288	279	229
Residual HCO_3^--CO_2	295	188	294
Total	583	467	523
Moles of acid per mole of glucose	2.57	2.49	2.07

cally from the glucose, the sum of CO_2 liberated to the gas phase and residual bicarbonate would have exceeded the initial HCO_3^--CO_2 by an amount equal to that of the metabolic CO_2.

Two to 2.5 moles of acid were produced per mole of glucose by the Salmonella. This suggests that a coli-type fermentation occurred with the formation of a mixture of organic acids and is in accord with the analytical data of Harden (1901), Scheffer (1928), Tasman and Pot (1934), and Friedemann (1938), on the fermentation of glucose by Salmonella.

If, however, the hydrogenlyase-deficient agar cells are incubated with a mixture of formate, glucose, and casein hydrolyzate, they adaptively form hydrogenlyase. The enzyme is first detectable, by the formation of H_2 in the vessels, about 30 minutes after the formate and cells are brought together and maximum activity is reached about 15 minutes later. The course of the adaptive process in *S. typhimurium* is shown in figure 5. When used singly, the formate, glucose, and casein hydrolyzate fail to stimulate enzyme formation. Occasionally glucose will permit some adaptation, but this is apparently due to nitrogenous impurities carried over in the cell suspensions from the culture medium, since washed cells will not adapt with glucose alone. The combination of glucose and hydrolyzed casein is sufficient for enzyme synthesis. The further addition of formate merely speeds up the rate of adaptation slightly. The non-essentiality of formate is understandable since the fermentation of the glucose by the cells automatically gives rise to formate.

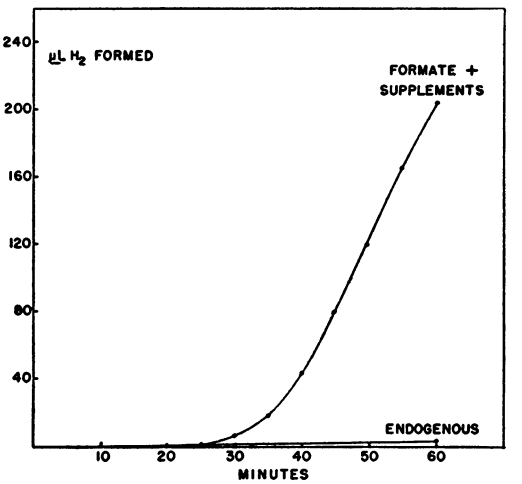

Figure 5. Adaptive formation of hydrogenlyase by *Salmonella typhimurium.*

Salmonella cells, like those of *E. coli*, therefore require both a source of energy and amino acids for the synthesis of hydrogenlyase. Also, as with *E. coli* (Pinsky and Stokes, 1952*a*) the optimum pH level for adaptation lies between pH 6 and pH 7 (figure 6). Synthesis of the enzyme is favored, therefore, by higher pH levels than the activity of the enzyme. The latter, it will be recalled, functions best at about pH 5.

The cells from agar, like those from broth, possess an active hydrogenase and with these cells, also, methylene blue is a better H_2 acceptor than fumarate. Hydrogen consumption with fumarate is very slow and with some strains

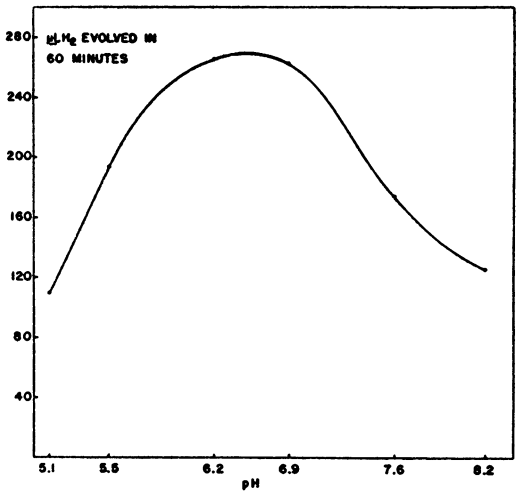

Figure 6. Influence of pH on hydrogenlyase adaptation in *Salmonella typhimurium.*

appears to be non-existent, since it may not exceed that of the endogenous control. In contrast, with methylene blue there is always an immediate and very rapid consumption of hydrogen.

Analyses for formic dehydrogenase were made only for *S. oranienburg* and *S. pullorum.* The enzyme appeared to be present in both organisms but only in small amounts.

TABLE 2

Distribution of enzymes in anaerogenic strains of Salmonella

Organism	Hydrogenlyase	Hydrogenase	Formic Dehydrogenase
S. gallinarum	−	+	+
S. typhimurium	−	+	−
S. montevideo	−	+	−
S. paratyphi B	−	+	+
S. oranienburg	−	+	+

Anaerogenic strains. With the exception of *S. typhosa* and *S. gallinarum,* strains of Salmonella which do not form gas from sugars are only infrequently encountered. From a biochemical point of view all of these anaerogenic cultures can be considered variants of the normal gas producing strains. Fortunately, we were able to obtain from various investigators anaerogenic strains of four typical gas producing Salmonella, *S. montevideo, S. typhimurium, S. paratyphi B,* and *S. oranienburg.* When cultured in tubes of glucose broth supplied with Durham vials, there was no evidence of gas formation with any of the strains even on prolonged incubation, although all of them formed acid. These strains and also *S. gallinarum,* which can be considered to be an anaerogenic variant of *S. pullorum,* were subjected to enzymatic analyses in order to compare them with the gas-forming strains.

The results of the enzymatic analyses of the anaerogenic cells harvested from glucose broth cultures are given in table 2. Unlike gas-forming strains, the cells did not produce H_2 from either formate or glucose. They do not contain hydrogenlyase, therefore, and resemble in this respect the agar-grown cells of the gas-forming strains previously described. But unlike the latter, the anaerogenic cells will not synthesize hydrogenlyase when placed in contact with formate, glucose, and casein hydrolyzate. They possess hydrogenase, which can be demonstrated with both fumarate and methylene blue and the latter, as usual, is the better hydrogen acceptor. Some of the strains exhibited strong formic dehydrogenase activity while others were completely inactive.

The absence of hydrogenlyase in the anaerogenic strains suggested that fermentations of glucose by them should lead to an accumulation of formic acid, whereas fermentations of hydro-

TABLE 3

Comparison of the properties of gas-forming and non-gas-forming strains of Salmonella

Strain Type	Acid	Gas	Hydrogenylase	Hydrogenlyase Adaptation	Hydrogenase	Formic Dehydrogenase	Accumulation of Formic Acid
Gas formers							
Broth cells	+	+	+		+	±	−
Agar cells	+	−	−	+	+	+	+
Non-gas formers							
Broth cells	+	−	−	−	+	+ or −	+

genlyase-positive, gas-forming strains should yield little or no formic acid. Analyses for this acid were made on glucose broth cultures of all nine Salmonella strains. Ten-ml portions of two-day-old cultures were acidified to pH 2 and steam distilled until 200 ml of distillate had been collected. The distillate was adjusted to pH 8, concentrated by boiling to about 10 ml, and analyzed for formic acid. The division between the strains was sharp. Formic acid was found in the cultures of all of the anaerogenic strains and was absent from the cultures of all of the gas-forming strains.

For ease of comparison, the pertinent data on the gas-forming and anaerogenic strains of Salmonella have been collected in table 3.

DISCUSSION

The principal conclusions that can be drawn from the foregoing data are of interest from the standpoint of comparative biochemistry. Gas formation from carbohydrates by *Salmonella*, as in the case of *Escherichia*, *Aerobacter*, and *Proteus*, is completely dependent upon the presence of formic hydrogenlyase in the cells. Gas-producing strains can synthesize this necessary enzyme, whereas anaerogenic strains cannot. Furthermore, the O_2, pH, energy, and amino acid requirements for hydrogenlyase formation in the genus *Salmonella* are similar and perhaps identical to those for *Escherichia*. The genus *Aerobacter* and especially *Proteus* have not been so extensively investigated, as yet, but there is no reason to suspect, on the basis of the available data, that they differ materially from the other two groups. These four genera have many morphological and biochemical properties in common and this close relationship is further emphasized by the similarities in their enzymatic and biochemical mechanisms for gas production.

There is much indirect evidence that the splitting of formic acid into H_2 and CO_2 may be due to the combined action of hydrogenase and formic dehydrogenase plus an intermediary electron carrier (Ordal and Halvorson, 1939; Gest, 1954). Recently, more direct evidence was supplied by Gest and Peck (1955) which indicates that probably two electron carriers are involved, in addition to the two enzymes. On the basis of the available information, it appears to us that a third enzyme must participate in the hydrogenlyase reaction. This conclusion stems from the fact that aerobically grown cells of gas-forming strains of Salmonella and also the coli-aerogenes group which contain both hydrogenase and formic dehydrogenase still must form, adaptively, an additional enzyme before they can liberate H_2 from formate. Whether this third adaptive enzyme participates directly in the hydrogenlyase reaction or indirectly by catalyzing the formation of an intermediate electron carrier remains to be determined. The possibility that the splitting of formic acid is due solely to the activity of the adaptive enzyme cannot yet be entirely excluded.

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SUMMARY

The formation of gas from carbohydrates by the genus *Salmonella* is due to the action of formic hydrogenlyase. The properties of this enzyme system in Salmonella are very similar to those of hydrogenlyase in *Escherichia coli*. Like the latter, aerobically grown cells do not contain hydrogenlyase but will rapidly and adaptively form the enzyme or enzyme system when supplied with both glucose and amino acids. The enzyme is most active at about pH 5 but is formed best, adaptively, by cell suspensions at pH 6 to pH 7. In contrast, anaerogenic strains of Salmonella neither synthesize hydrogenlyase during growth in glucose broth nor adaptively in resting-cell suspensions with supplements. As a consequence, formic acid accumulates in glucose fermentations by the anaerogenic strains. This does not occur with gas-forming strains.

Cells of both gas-forming and non-gas-forming strains of Salmonella contain an active hydrogenase. Hydrogen consumption by cell suspensions could invariably be demonstrated with methylene blue as hydrogen acceptor, but not always with fumarate as acceptor. Formic dehydrogenase was present, unequivocally, in some of the gas-forming and anaerogenic strains. The data indicate that there must be an enzyme other than hydrogenase or formic dehydrogenase which participates in the hydrogenlyase reaction.

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