A COMPARISON OF HYDROGEN PRODUCTION FROM SUGARS AND FORMIC ACID BY NORMAL AND VARIANT STRAINS OF ESCHERICHIA COLI[1]

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It is common knowledge that the gases formed by members of the colon-typhoid group of bacteria consist of mixtures of hydrogen and carbon dioxide. Pakes and Jollyman (1901) showed that pure cultures of organisms of this group produced mixtures of hydrogen and carbon dioxide from formic acid. Since they also produced these gases from glucose, Pakes and Jollyman concluded that the gas from glucose came from intermediately-formed formic acid. Harden (1901) showed that the most striking difference in the fermentation reactions of *Escherichia coli* and *Eberthella typhosa* is that the latter is unable to decompose formic acid to hydrogen and carbon dioxide. He made the further observation that increased pressure decreased the production of hydrogen and carbon dioxide and increased the amount of formic acid in the fermentation of glucose by *Escherichia coli.* The latter observation suggested the reversibility of the reaction, later demonstrated by Woods (1936).

It has generally been believed by most investigators of fermentations by the colon-typhoid group that the hydrogen formed during fermentation comes from intermediate formic acid. Most fermentation schemes, therefore, (e.g. Kluyver, 1931), designate formic acid as the precursor of the hydrogen formed.

However, Stephenson and Stickland (1932) and Stephenson

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(1937) present evidence that hydrogen may be produced by *Escherichia coli* from glucose and other carbohydrates, otherwise than from preliminarily-formed formic acid. Tasman and Pot (1935) could not confirm these findings.

Quastel and Whetham (1925), using the Thunberg methylene-blue technique, showed that *Escherichia coli,* which produces hydrogen and carbon dioxide from glucose, is able to reduce methylene blue in the presence of formic acid. Early studies in this investigation showed that certain strains of *Escherichia coli,* which had lost the ability to produce gas from sugars and sodium formate, had also lost the ability to reduce methylene blue with formic acid. This observation supported the suggestion that formic acid is the precursor of hydrogen and carbon dioxide in the fermentation of glucose by *Escherichia coli.*

However, Stickland (1929) showed that *Eberthella typhosa* which does not produce gas from glucose, is able to reduce methylene blue in the presence of formic acid. Consequently, factors other than the activation of formic acid must be operative in order that carbon dioxide and hydrogen be produced.

Since the work of Quastel, various investigators have studied the enzymes of bacteria of the colon-typhoid group which liberate gases from sugars and formic acid. Quastel (1925) described one of these as formic dehydrogenase, catalysing the reaction $HCOOH + R \rightleftharpoons RH_2 + CO_2$ where R is methylene blue or some other hydrogen acceptor. Another enzyme, hydrogenase, was studied by Tauss and Donath (1930) and Stephenson and Stickland (1931). Green and Stickland (1934) have shown that hydrogenase catalyses the reaction $H_2 \rightleftharpoons 2H^+ + 2(e)$ in a perfectly reversible manner.

Stephenson and Stickland (1932) have given the name "formic hydrogenlyase" to an enzyme which liberates hydrogen and carbon dioxide from formic acid, catalysing the reaction $HCOOH \rightleftharpoons H_2 + CO_2$. They are of the opinion that formic hydrogenlyase cannot be regarded as a combination of formic dehydrogenase and hydrogenase. Furthermore, they present evidence that hydrogenlyases other than formic hydrogenlyase, in particular glucose hydrogenlyase, exist.

From the work of Dienert (1900) and Karström (1938) it appears that the enzymes concerned in the fermentation of carbohydrates by bacteria may be of two types. Enzymes of the first type, called adaptive enzymes by Karström, appear in active form only when suitable substrates are present in the medium on which the organisms are grown. Enzymes of the second type, constitutive enzymes, are present independently of the substrates upon which they act.

Stephenson and Stickland (1933) have shown that formic hydrogenlyase can be classified as an adaptive enzyme. Organisms grown in broth alone do not produce gas from formic acid. However, the addition of sodium formate or glucose to growing cultures leads to the production of formic hydrogenlyase. The production of this enzyme does not occur in response to natural selection, nor is it necessarily accompanied by any considerable cell multiplication. This work is of special interest in view of the fact that some of the data here presented show that enzymes can be permanently or temporarily lost, and that the loss involved is a different phenomenon from the absence of an adaptive enzyme that may be regenerated by the addition of a suitable substrate.

The basis of this investigation is a comparison of the enzymes of normal *Escherichia coli*, with those of anaerogenic variants, in an effort to determine the nature of the enzymes concerned in the production of hydrogen from sugars and formic acid.

Numerous investigators have reported the occurrence of such anomalous strains. Neisser (1906) described *Bacillus coli-mutabile*, an organism that did not produce acid from lactose but consistently produced variants able to ferment that sugar. Penfold (1912) reported strains of *Eberthella typhosa* that failed to ferment dulcitol but gave rise to dulcitol-fermenting strains. Revis (1912) reported that strains of *Escherichia coli*, trained to tolerate malachite green, lost the ability to form gas on sugars. Penfold (1911) observed the same phenomenon with organisms grown with chloracetic acid. Similar phenomena have been reported by other investigators.

EXPERIMENTAL

The production of biochemical variants by a strain of Escherichia coli

Several years ago a strain of *Escherichia coli* (*communis*), from the collection at the University of Minnesota, was cultivated for use in testing germicides. Several transplants were made, and these were subcultured serially on nutrient broth containing Armour's peptone. The cultures were regularly plated and checked on glucose, maltose, lactose, sucrose, and mannitol broths. It was soon observed that certain cultures no longer produced gas on carbohydrates. Repeated plating of these cultures yielded a number of strains that behaved normally and a number that failed to produce gas on carbohydrate. Several of these variants were selected for further study. On repeated subculturing and plating, some reverted to gas production; others remained stable. Subculturing on sodium formate or carbohydrate broth favored reversion to gas production; however, some variants remained stable in spite of cultivation on carbohydrate medium.

All variants which failed to produce gas in sodium formate broth also failed to do so as from the usual carbohydrates. However, when variants reverted to gas production in sodium formate, they were then also able to produce gas from glucose and other carbohydrates. This point was checked repeatedly in view of the opinion expressed by Stephenson and Stickland (1933) and Stephenson (1937) that hydrogen may be produced from glucose without the intermediate production of formic acid, and that the enzymes responsible for the production of hydrogen from glucose and formic acid are distinct and separate. All cultures that produced gas from glucose also produced gas from sodium formate, and vice versa.

The colonies that appeared on plating were smooth, rough or intermediate in form. A large number of representative colonies were picked and tested for gas production. However, there was no correlation between roughness or smoothness of colony and ability to produce gas on carbohydrate broths. This was further confirmed by plating on deep agar.

Although most of the variants differed from the normal organism only in not being able to produce gas, we did encounter some variants that had lost the power to produce acid as well as gas from maltose. Cultivation of these on sugar broths favored reversion; however, stable strains were obtained which remained unable to produce acid on maltose even after serial transfer on maltose broth for several months.

Methods employed in the study of the enzymes of normal Escherichia coli and its variants

In this investigation, the enzymes of these organisms have been studied by the Thunberg methylene-blue technique as used by Quastel, or a modification of it; by measurement of pH changes in growing cultures to which various substrates were added; and by measurements of gas evolution with Warburg manometers.

Methylene blue technique. The organisms were grown regularly on nutrient broth containing beef extract, peptone and water. Carbohydrate broths contained in addition 0.5 per cent of stock sugar and Andrade's indicator. The medium used for the production of mass cultures was buffered by the addition of 0.5 or 1 per cent of disodium phosphate. All organisms used in enzyme studies were transferred daily for several days on broth before inoculation into the mass culture flask. Organisms were regularly checked on routine media.

The sugar solutions were sterilized by filtration through Berkefeld or Seitz filters, and added in appropriate amounts to the mass culture medium. Following inoculation, the cultures were incubated twenty-four to forty-eight hours at 38°C. Mass cultures were repeatedly centrifuged and resuspended in saline or Ringer solutions. The organisms must be washed carefully because the presence of extraneous organic matter will influence the results of the experiments. Since, in these experiments, the organisms catalyse reduction of methylene blue in the presence of specific substrates, it is necessary that other substances present in the culture which can act as hydrogen donators be removed so far as possible. Organisms such as *Aerobacter aerogenes* secrete capsular material which is difficult to remove by washing,

and frequently there is enough of this extraneous material in the culture to mask the reaction of the substrate.

Morphological variants which produce a firm pellicle or grow in granular form are difficult to suspend and reactions of such cultures are slow on all substrates. Except in the earlier experiments, suspensions were standardized by compacting in calibrated centrifuge cups, and resuspending the sediment in twenty times its volume of saline.

The earlier experiments were performed in tubes of the Thunberg type, in each of which were placed the suspension of organisms, a buffer, an oxidation-reduction indicator such as methylene blue, and the substrate to be tested. The tubes were evacuated, filled with oxygen-free nitrogen, and placed in a water bath. The time for decolorization of the indicator was determined by visual observation.

In the later experiments, a modification of the Thunberg technique was used. The test tubes were equipped with side arms containing soft rubber stoppers, such as are used on vaccine bottles. Each tube was closed with a two-hole rubber stopper providing an inlet and an outlet for the gas (oxygen-free nitrogen saturated with water), which was bubbled through the liquid in the tube. Oxygen was removed from the nitrogen by passage over hot copper gauze in a Kendall (1931) apparatus. The tubes were kept in a water bath at a definite temperature, usually 30°C. After the removal of entrapped air and the attainment of the proper temperature within the tube, the suspension of organisms was injected by means of a hypodermic syringe through the soft rubber stopper in the side arm. The decolorization of methylene blue was followed by visual comparison with control tubes, left open to the atmosphere, from which substrates had been omitted, and which contained varying amounts of methylene blue. Such comparisons showed the relative amounts of methylene blue that had been reduced, and facilitated the determination of the final end point.

These reduction experiments were controlled by setting up tubes containing organisms and methylene blue with no substrate. In order that the results be significant, it is necessary that the control tubes do not decolorize throughout the experi-

ment. There must be a significant difference in time of decolorization in the presence and in the absence of substrate. Using fresh suspensions, the methylene blue in the control tubes remained in the oxidized form for several hours, while with older suspensions, reduction might occur in a relatively short time.

Changes in pH of cultures. The same basic medium was employed in these studies as was used in the production of mass cultures. To this basic medium was added glucose, maltose or sodium formate solution that had been sterilized by filtration through Seitz or Berkefeld filters. After the addition of the carbohydrate, the medium was incubated for 48 hours at 37°C. to test for sterility. After inoculation, the cultures were again incubated for 48 hours. The pH of both the cultures and the uninoculated broth was determined at 30°C. by means of a glass electrode.

Warburg technique. The suspensions used were prepared as described above. Warburg manometers (Dixon, 1934) with nearly uniform cups of 20 ml. capacity were used. Into each cup 1 ml. of buffer, 1 ml. of 0.1M substrate, and 0.5 ml. of washed suspension of cells to be tested were introduced by means of pipettes; 0.3 ml. of a 30 per cent solution of potassium hydroxide was placed in the inner cup for the absorption of carbon dioxide. A stream of nitrogen purified by passage over hot copper was led through the manometer for ten minutes. The manometer was then placed in a constant temperature water bath at 30°C. and shaken with a 4 cm. stroke at a rate of 120 oscillations per minute. Ten minutes after placing the manometer in the thermostat, the manometric liquid was set at 0 and the stopcock closed. The evolution of gas was determined by measuring the rise of the manometer fluid in the open arm after adjusting the fluid in the closed arm to the zero point. Readings were therefore taken at constant volume. Manometers containing no substrate but the same volume of liquid were used as thermobarometric controls.

Reduction tests with normal and variant E. coli

The earlier experiments of this nature were performed with Thunberg tubes following the technique of Quastel (1932). Thunberg tubes contained the ingredients as listed in the tables.

The tubes were evacuated by a motor-driven air pump and placed in a water bath at 45°C. The time of decolorization was noted. In cases where decolorization proceeded slowly, the experiments were terminated before all the tubes were decolorized. In the first experiments reported, that amount of bacterial suspension was used which would give decolorization in approximately ten minutes with 0.01M sodium succinate.

Table 1 shows a comparison of the activation of sodium formate and sodium succinate by culture I, a normal *Escherichia coli*,

TABLE 1

Comparison of activation of sodium formate by cultures I and V

CULTURE	I	I	I	V	V	V
Tube number	1	2	3	4	5	6
1/5000 Methylene blue (ml.)	1	1	1	1	1	1
M/20 Sodium succinate (ml.)	0	0	1	0	0	1
M/15 Phosphate buffer, pH 7.5 (ml.)	1	1	1	1	1	1
M/10 Sodium formate (ml.)	0.5	0	0	0.5	0	0
Suspension of organism (ml.)	0.5	0.5	0.5	0.4	0.4	0.4
Water (ml.)	2	2.5	1.5	2.1	2.6	1.6
Total volume (ml.)	5	5	5	5	5	5
Time of decolorization (min.)	3.5	>120	13.5	>120	>120	10.6

with that of culture V, a variant unable to produce gas from sugars and sodium formate.

The two suspensions are comparable in their ability to reduce methylene blue with 0.01M sodium succinate. However, the variant culture was unable to reduce methylene blue in the presence of 0.01M sodium formate, whereas the normal organism did this in 3.5 minutes. The experiment was confirmed, using five tubes for each test.

In the normal fermentation of carbohydrates by *E. coli*, gas appears in a pH range of approximately 4.5 to 7.5. It was therefore considered desirable to investigate the activation of sodium formate by suspensions of normal and variant strains throughout this range.

Tables 2 and 3 illustrate the effect of pH on the time of decolorization with 0.01M sodium formate by cultures I and V. The suspension used in these experiments was not the same as that used in Experiment I; hence corresponding tubes do not show the same reduction time with 0.01M sodium succinate.

TABLE 2

Effect of pH on activation of sodium formate by culture I

Tube Number	1	2	3	4	5	6	7	8
1/5000 Methylene blue (ml.)	1	1	1	1	1	1	1	1
M/10 Sodium formate (ml.)	0.5	0.5	0.5	0.5	0.5	0.5	0	0
M/15 Phosphate buffer (ml.)	1	1	1	1	1	1	1	1
pH	7.5	6.9	6.5	5.9	5.2	4.4	7.2	7.2
M/20 Sodium succinate (ml.)	0	0	0	0	0	0	1	0
Suspension of organism (ml.)	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7
Water (ml.)	1.8	1.8	1.8	1.8	1.8	1.8	1.3	2.3
Total volume (ml.)	5	5	5	5	5	5	5	5
Time of decolorization (min.)	3	2.5	3	3	3.5	4	10	60

TABLE 3

Effect of pH on activation of sodium formate by culture V

Tube Number	1	2	3	4	5	6	7	8
1/5000 Methylene blue (ml.)	1	1	1	1	1	1	1	1
M/10 Sodium formate (ml.)	0.5	0.5	0.5	0.5	0.5	0.5	0	0
M/15 Phosphate buffer (ml.)	1	1	1	1	1	1	1	1
pH	7.5	6.9	6.5	5.9	5.2	4.4	7.2	7.2
M/20 Sodium succinate (ml.)	0	0	0	0	0	0	1	0
Suspension of organism (ml.)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Water (ml.)	2	2	2	2	2	2	1.5	2.5
Total volume (ml.)	5	5	5	5	5	5	5	5
Time of decolorization (min.)	60	70	>90	>90	>90	>90	10	64

It is evident that variant culture V was unable to activate sodium formate to reduce methylene blue throughout this range of pH. Decolorization of the control tube containing no substrate at pH 7.2 indicated that the cells had not been washed free of oxidizable matter.

The increase in time required for decolorization with increasing acidity, as shown in table 3, may be due to the effect of pH on the extraneous substances produced by the cells. Tube 8, a control with no substrate added, was reduced in a time intermediate between the times of reduction in tubes 1 and 2 containing sodium formate, the tubes most nearly corresponding in pH. The cells in this experiment were not fresh, as indicated by the reduction time for tube 8, a control.

Culture VII, a variant, and culture VIII, producing gas from carbohydrates and sodium formate, were isolated from the same

TABLE 4

Comparison of activation of sodium formate by cultures VII and VIII

CULTURE	VII	VII	VII	VIII	VIII	VIII
Tube number	1	2	3	4	5	6
1/5000 Methylene blue (ml.)	1	1	1	1	1	1
M/20 Sodium succinate (ml.)	0	0	1	0	0	1
M/10 Sodium formate (ml.)	0.5	0	0	0.5	0	0
M/15 Phosphate buffer, pH 7.5 (ml.)	1	1	1	1	1	1
Suspension of organism (ml.)	1.2	1.2	1.2	1.7	1.7	1.7
Water (ml.)	1.3	1.8	0.8	1.8	2.3	1.3
Total volume (ml.)	5	5	5	5	5	5
Time of decolorization (min.)	>120	>120	13	3.7	>120	8

source, a variant culture which reverted to gas production after continued cultivation on carbohydrate media. Since it was possible that gas production might depend on more than one factor, it was of interest to determine whether the property of activation of sodium formate was present in these cultures.

This experiment showed that culture VIII activated sodium formate strongly, whereas culture VII, from the same parent culture, did not activate sodium formate. Previous testing of the parent culture had shown that the ability to activate sodium formate was absent.

In the above experiments the mass cultures employed were grown on nutrient broth. In all experiments with variant or-

ganisms, the ability to activate sodium formate to reduce methylene blue had been found lacking, whereas this ability had always been found in the normal organism. The question then arose as to whether the enzyme which activates formate is an adaptive enzyme which is lacking in the variant because of absence of ingredients stimulating its formation in the medium employed in growing the mass cultures.

Yudkin (1932) and Stephenson and Stickland (1932) have shown that the enzymes concerned in gas production by *Escherichia coli* may be considered to be adaptive. It was therefore necessary to grow the normal and variant cultures on media which might stimulate the production of such enzymes.

In the next series of experiments, normal and variant organisms were tested for action on sodium formate and molecular hydrogen when grown on phosphate-buffered broth, and on phosphate broth containing glucose or maltose.

The modified methylene-blue technique described earlier was used. Suspensions of washed organisms were standardized by centrifuging in graduated tubes and made up to the desired volume, usually twenty times the volume of the organisms.

A considerable number of strains of normal and variant *Escherichia coli* were tested. Typical results are shown in table 5. The reduction experiments were terminated at sixty minutes; lack of decolorization of methylene blue at this time is indicated by >60′.

It is apparent from table 5 that culture II, normal *Escherichia coli*, can activate sodium formate and molecular hydrogen to reduce methylene blue regardless of the presence or absence of glucose or maltose in the medium used for growth. A considerable number of strains of *Escherichia coli* isolated from sewage were tested for their ability to reduce methylene blue with sodium formate and with molecular hydrogen. All possessed active enzymes, irrespective of the medium used for growth. Thus the enzymes concerned in the activation of hydrogen and sodium formate must, according to Karström, be considered constitutive. Cultures of anaerogenic variants which had reverted to gas production were tested for these enzymes (see

table 4). In every case organisms able to produce gas were found to possess hydrogenase and formic dehydrogenase.

The various types of anaerogenic variants found are shown in table 5. The activities of the enzymes, formic dehydrogenase and hydrogenase, varied considerably in different strains, but did not appear to be dependent upon the presence of glucose or maltose in the medium used for growth. Most of the variants

TABLE 5

The activation of sodium formate and molecular hydrogen by normal and variant Escherichia coli

Atmosphere	Nitrogen						Hydrogen		
Medium used for growth	b.	g.b.	m.b.	b.	g.b.	m.b.	b.	g.b.	m.b.
1/5000 Methylene blue (ml.)	1	1	1	1	1	1	1	1	1
M/15 Phosphate buffer, pH 6.9 (ml.)	5	5	5	5	5	5	5	5	5
M/10 Sodium formate (ml.)	2	2	2	0	0	0	0	0	0
Water (ml.)	0	0	0	2	2	2	2	2	2
Suspension of organisms (ml.)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Total volume (ml.)	8.5	8.5	8.5	8.5	8.5	8.5	8.5	8.5	8.5
	Time of decolorization in minutes								
E. coli II	2	3.4	3	>60	>60	>60	6.2	6.2	4.2
Variant X	>60	>60	>60	>60	>60	>60	16	4	8
Variant XI	1.2	2.2	1.7	>60	>60	>60	>60	>60	>60
Variant XII	2.2	6.7	3.5	>60	>60	>60	3	4.5	3
Variant XIII	>60	>60	>60	>60	>60	>60	44	>60	54

b., phosphate broth; g.b., glucose phosphate broth; m.b., maltose phosphate broth.

tested were of the type of Variant X, that is, they were unable to activate formate. In only one case was an anaerogenic culture (Variant XII) found which was able to activate both hydrogen and formate, i.e., possessed both hydrogenase and formic dehydrogenase. It is apparent from the existence of this culture that some factor other than the presence of these enzymes is necessary for production of gas. Further evidence to this effect arises from the fact that a suspension of normal *Escherichia coli* grown in broth without proper substrate produces little or no

gas from glucose or sodium formate, whereas gas is produced at a rapid rate if sugars are present in the medium used for growth.

Stephenson and Stickland (1932) and Stephenson (1937) have concluded that formic hydrogenlyase, the enzyme concerned in the production of gas from sodium formate, is an enzyme separate and distinct from formic dehydrogenase and hydrogenase. This is in opposition to their earlier view (Stephenson and Stickland 1931) that formic dehydrogenase and hydrogenase together make up the enzyme producing gas from formic acid. Their conclusion was based in part on evidence that *Bacillus lactis-aerogenes* possessed no hydrogenase, but was able to produce gas from formic acid, i.e., possessed formic hydrogenlyase. In their earlier paper Stephenson and Stickland considered the presence of hydrogenase in *B. lactis-aerogenes* questionable since reduction with hydrogen in Thunberg tubes took place in 9½ minutes, whereas reduction occurred in 15½ minutes in the control. In their second paper, this question was reëxamined, and the conclusion reached that the enzyme was absent, but no conclusive data were given.

It seemed, therefore, desirable to test cultures of *Aerobacter* species for the presence of hydrogenase.

Hydrogenase in aerobacter species

Three strains of *Aerobacter aerogenes* and two strains of *Aerobacter cloaceae* were subcultured in broth containing 0.25 per cent sodium formate and 0.25 per cent glucose every three days for a month. Mass cultures were then grown in broth containing 1 per cent phosphate, 0.25 per cent sodium formate and 0.25 per cent glucose. The organisms were harvested at 36 hours and washed three times in 0.9 per cent saline. The suspensions were then tested for hydrogenase, using the modified methylene blue method. The results are shown in table 6.

Suspensions of *Aerobacter aerogenes* 401 were further tested for hydrogenase in solutions of varying pH, as shown in table 7.

It is clear that the suspension of *Aerobacter* species tested reduced methylene blue more rapidly in the presence of hydrogen than in the presence of nitrogen. In relatively concentrated

suspensions, the reducing matter associated with the cells masked the presence of hydrogenase. The effect of change in pH shows that more than one factor is operating in those tests where hydro-

TABLE 6

Tests for hydrogenase in aerobacter species

CULTURE	SUSPENSIONS OF ORGANISMS USED					
	1:20		1:40		1:100	
	Times of reduction of methylene blue in minutes in atmospheres of					
	H_2	N_2	H_2	N_2	H_2	N_2
Aerobacter aerogenes 401	2.0	18	2.7	51	10	>90
Aerobacter aerogenes 403	2.7	10.4	4.4	32	16	>90
Aerobacter aerogenes 404	2.3	10.8	7.1	>90		
Aerobacter cloacae 451	2.8	5.1	6	13	12	>90
Aerobacter cloacae 452	4	61				

Suspensions of organisms are from mass cultures grown for 36 hours at 37°C. on 1 per cent phosphate broth with 0.5 per cent glucose and 0.25 per cent sodium formate.

Each tube contained: 5 ml. M/15 phosphate buffer, pH 6.9, 0.5 ml. 1/5000 methylene blue, 0.5 ml. suspension of organisms.

Temperature of experiment: 30°C.

TABLE 7

The effect of pH and concentration of cells on hydrogenase in aerobacter aerogenes

pH	SUSPENSIONS OF ORGANISMS USED					
	1:20		1:40		1:100	
	Times of reduction of methylene blue in minutes in atmospheres of					
	H_2	N_2	H_2	N_2	H_2	N_2
6.3	3.2	20	4.5	63	18	>120
6.7	2.5	18.5	3	51	13.5	>120
7.1	1.8	17.5	2.5	53	10.5	>120
7.5	2	17	3	48	8.2	>120
7.8	3	12.5	3.2	47	8	>120

Suspensions from a mass culture grown for 36 hours at 37°C. on 1 per cent phosphate broth with 0.5 per cent glucose and 0.25 per cent sodium formate.

Each tube contained: 5 ml. M/15 phosphate buffer, 0.5 ml. 1/5000 methylene blue, 0.5 ml. suspension of organisms.

Temperature of experiment: 30°C.

gen was used. Hence it must be concluded that these *Aerobacter* species possess hydrogenase.

TABLE 8

Final pH values of media inoculated with normal and variant strains of E. coli

ORGANISM	REACTION ON GLUCOSE BROTH (Using Durham tubes)	REACTION ON MALTOSE BROTH (Using Durham tubes)	BROTH	GLUCOSE BROTH	MALTOSE BROTH	FORMATE BROTH
			pH	*pH*	*pH*	*pH*
Uninoculated			7.1			
E. coli II	A G	A G	7.2	4.7	5.0	8.1
Variant X	A —	A —	7.1	4.8	4.8	7.1
Variant XI	A —	— —	7.1	4.8	7.0	7.0
Variant XII	A —	A —	7.1	4.7	5.2	7.0
Variant XIII	A —	— —	7.2	5.4	7.0	7.1

TABLE 9

Hydrogen evolution by Escherichia coli from sodium formate, glucose and maltose

SUBSTRATE	MEDIUM USED FOR GROWTH	GAS EVOLVED IN MM³ AT (TIME IN MINUTES) 0	5	10	15	20	30	45	60	90
Formate	b.	0	−1	−1	−2	−2	−1	−1	0	1
	g.b.	0	7	16	24	33	52	80	109	165
	m.b.	0	53	111	145	187	255			
Glucose	b.	0	0	1	2	5	11	28	57	135
	g.b.	0	5	9	16	23	41	68	97	158
	m.b.	0	15	33	52	68	108	165	223	
Maltose	b.	0	−1	−1	0	2	6	19	35	76
	g.b.	0	4	8	13	17	28	52	86	159
	m.b.	0	26	57	101	153	225	294		

b., phosphate broth; g.b., glucose phosphate broth; m.b., maltose phosphate broth.

Warburg cups contains: 1 ml. pH 6.9 M/15 phosphate buffer, 1 ml. 0.1M substrate, 0.5 ml. suspension of organism.

Inner cup contains: 0.3 ml. 30 per cent potassium hydroxide.

Temperature of experiment: 30°C.

Changes in pH by normal and variant Escherichia coli

The pH values were determined in cultures of normal and variant *Escherichia coli* grown at 37°C. for 48 hours in 1 per cent

phosphate broth, and in phosphate broth containing 0.5 per cent glucose, 0.5 per cent maltose, or 0.5 per cent sodium formate.

It will be seen that the variant cultures producing no gas from sugars and formate are inert towards sodium formate, whereas the normal culture produces a pronounced alkalinity in formate broth. Furthermore, the variant cultures which, according to the Durham tube criterion, are unable to produce acid from maltose do not change the pH appreciably in the presence of maltose.

Hydrogen evolution by normal and variant Escherichia coli

Suspensions of normal and variant *Escherichia coli* were tested for production of hydrogen from formic acid, glucose and maltose in Warburg manometers.

The variant cultures tested for hydrogen production were negative in all cases, confirming their anaerogenic character indicated by the usual carbohydrate media. The results obtained with the normal strain of *Escherichia coli* are given in table 9.

The suspension grown on broth produced no hydrogen from sodium formate, but produced gas from glucose and maltose after a latent period. Suspensions grown in glucose or maltose broths produced gas at once from sodium formate, glucose or maltose.

DISCUSSION

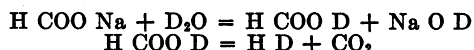
It is highly probable that the hydrogen produced from sugars by *E. coli* comes from intermediately-formed formic acid. Variant strains of *E. coli* which fail to form gas from sugars are unable to produce gas from formate. Variant strains reverting to the aerogenic form, following cultivation on sugar broths, produce gas from formate as well as from sugars.

Anaerogenic variants of *E. coli* may or may not possess the enzymes hydrogenase and formic dehydrogenase in active form. However, on reversion to the aerogenic form, these enzymes appear in active form and their presence is independent of the presence of glucose or maltose in the medium used for growth. Hydrogenase and formic dehydrogenase have been found in all strains of *E. coli* capable of producing gas.

The determination of the presence of hydrogenase in *Aerobacter* species makes tenable the theory that the production of hydrogen and carbon dioxide from formic acid is the result of the combined action of the enzymes hydrogenase and formic dehydrogenase, and makes unnecessary the postulation of formic hydrogenlyase as an enzyme separate and distinct from, and not composed of hydrogenase and formic dehydrogenase.

Farkas, Farkas and Yudkin (1934), studying the decomposition of sodium formate by *Escherichia coli* in heavy water of varying deuterium content at pH 7, have shown that the hydrogen evolved by the decomposition of formate, with regard to its deuterium content, was in equilibrium with the water in the solution from which it was liberated. Analysis of the hydrogen evolved with regard to the three different molecular species H_2, HD and D_2 showed that these three constituents were present in their equilibrium concentration. In addition, the observation was made that if normal water was left in contact with hydrogen containing 30% D in the presence of *Escherichia coli*, a complete replacement of the deuterium gas by normal hydrogen occurred in a few hours.

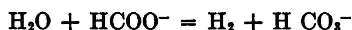
They concluded that reactions of the type:



$$\mathrm{H\,COO\,Na} + \mathrm{D_2O} = \mathrm{H\,COO\,D} + \mathrm{Na\,O\,D}$$
$$\mathrm{H\,COO\,D} = \mathrm{H\,D} + \mathrm{CO_2}$$

could not express the decomposition of formate. Since only the hydrogen atom in the acid radicle can be replaced by D, D_2 can not be formed according to this reaction.

Krebs (1937), on the basis of the experiments performed by Farkas, Farkas and Yudkin, suggested that the mechanism of decomposition of formate can be represented by the following equation:



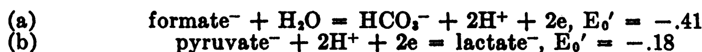
$$\mathrm{H_2O} + \mathrm{HCOO^-} = \mathrm{H_2} + \mathrm{H\,CO_3^-}$$

where the formate is oxidized by the water, accompanied by a liberation of hydrogen. According to this view, the hydrogen evolved is derived from the water of the medium, and is therefore in equilibrium with the water in respect to deuterium content in the experiments quoted above.

Borsook (1935) studied the reaction:

$$\text{pyruvate}^- + \text{formate}^- + H_2O = \text{lactate}^- + HCO_3^-$$

in the presence of toluene-treated suspensions of E. coli. The components of this reaction may be written:



$$\text{(a)} \quad \text{formate}^- + H_2O = HCO_3^- + 2H^+ + 2e, \; E_0' = -.41$$
$$\text{(b)} \quad \text{pyruvate}^- + 2H^+ + 2e = \text{lactate}^-, \; E_0' = -.18$$

It was discovered that pyruvate and formate do not react at a significant rate in the presence of toluene-treated organisms unless a dye is present whose E_0' is intermediate between that of the lactate-pyruvate and formate-bicarbonate systems. The explanation given for the necessity of an intermediary dye system is that in the toluene-treated organisms, electron conduction cannot occur between the lactate-pyruvate and formate-bicarbonate enzymes.

Borsook's theory of complete and incomplete enzyme centers may well be applied to the decomposition of formate by *E. coli.* A suspension of *E. coli* grown in broth alone is unable to produce gas from formate, and is also unable to reverse the reaction and synthesize formate from hydrogen and carbon dioxide (see Woods 1936), although the enzymes, formic dehydrogenase and hydrogenase, can be shown to be present. Our work shows that cells grown in broth containing sugars are able to produce gas from formate. According to Stephenson, this is also true of cells grown in broth containing formate. Because of this phenomenon the enzyme liberating gas from formate is said to be "adaptive." It is possible that in the suspension of *E. coli* grown in broth alone, some factor may be lacking so that electron conduction between hydrogenase and formic dehydrogenase cannot occur. On the other hand, cells grown on formate or carbohydrate broth produce gas from formate because this missing factor is present, and hence electron conduction can occur.

When we consider the synthesis of formate:

$$H_2 + HCO_3^- = H_2O + HCOO^-$$

it is evident that the enzyme system must be able to activate molecular hydrogen as well as bicarbonate. Since such a system

must therefore possess the combined characteristics of the two enzymes, hydrogenase and formic dehydrogenase, it seems reasonable to believe that the production of hydrogen and carbon dioxide from formate is the result of the combined action of these same enzymes.

The two components of the reaction:

$$H_2O + HCOO^- = H_2 + HCO_3^-$$

may be expressed as:

$$\text{(a)} \quad H_2O + HCOO^- = HCO_3^- + 2H^+ + 2e$$
$$\text{(b)} \quad 2H^+ + 2e = H_2$$

According to this scheme, the nature of the hydrogen evolved would necessarily be determined by the nature of the water. This is in agreement with the results of Farkas, Farkas and Yudkin in their experiments with heavy water.

From experiment IX it is apparent that resting cells which have been grown in plain broth are not able to regenerate the enzyme hydrogenlyase in the presence of formate, but are able to do so in the presence of glucose or maltose. According to Stephenson, organisms which have been grown in plain broth are able to produce the enzyme hydrogenlyase if they are subsequently cultured on broth containing formate. The fact that formate can bring about the regeneration of this enzyme in the one case where organisms are grown in its presence, and not in the other, indicates that the regeneration is controlled by factors other than the mere presence of formate. This question is being studied further, and will be discussed in greater detail in a later publication.

SUMMARY

Variant strains of *Escherichia coli* were isolated which produced no gas from formate and carbohydrate broths. Culturing in formate and carbohydrate broths favored reversion to the aerogenic form. In all cases, variants reverting to gas production produced gas from formate as well as from glucose and other carbohydrates.

No correlation was found between roughness and smoothness of colony and ability to produce gas.

A further variation, loss of power to form acid from maltose, occurred in some anaerogenic strains.

Anaerogenic variants have been found that possess both hydrogenase and formic dehydrogenase, others have been found that possess only one of these enzymes, and still others have neither of them. However, the enzymes are always present in cultures which have reverted to the aerogenic form. All normal strains of *Escherichia coli* were found to possess these enzymes in active form, irrespective of the presence or absence of glucose or maltose in the medium used for growth.

Three strains of *Aerobacter aerogenes* and two strains of *Aerobacter cloacae* were shown to possess the enzyme hydrogenase, although the presence of this enzyme may be masked by reducing systems associated with the bacterial cells.

The determination of pH changes in growing cultures showed that the anaerogenic variants were inert towards formate, and that the maltose variants produced no significant amount of acid from maltose.

All anaerogenic variants failed to produce hydrogen from maltose, glucose and formate when tested in Warburg manometers. Normal *Escherichia coli* grown in phosphate broth produced no gas from formate but did produce gas from maltose and glucose after a latent period. Suspensions of *Escherichia coli* grown in glucose or maltose broth produced hydrogen from formate, glucose or maltose at a rapid rate.

The results of our investigation indicate that the hydrogen produced from glucose by *Escherichia coli* comes from formic acid, which is an intermediate product in the fermentation of sugar, and that formic dehydrogenase and hydrogenase are constituents of the enzyme liberating gas from formic acid.

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