CXXXI. THE BACTERIAL DECOMPOSITION OF FORMIC ACID.

By LEONARD HUBERT STICKLAND.

From the Biochemical Laboratory, Cambridge.

(*Received October 31st, 1929.*)

THE bacterial decomposition of formic acid is a subject which, in spite of its apparent simplicity, still presents unsolved problems; it was in the hope of elucidating some of these that the present study was undertaken.

The earliest observation on the subject was made by Hoppe-Seyler [1875]. Later Pakes and Jollyman [1901] studied the anaerobic decomposition of formates by bacteria in a state of active multiplication in meat broth. They collected the mixture of gases formed day by day and analysed it, and, after estimating the carbon dioxide left in the medium, found that the ratio of hydrogen to carbon dioxide was exactly unity, indicating the simple reaction

$$H.COOH = H_2 + CO_2.$$

They did not estimate the amount of formic acid that disappeared, but state that, beginning with 0·2 % to 2·0 % formic acid, roughly 25–30 % was decomposed. From among upwards of thirty species of bacteria used, all those producing gas from glucose also decomposed formic acid anaerobically, and all those producing no gas failed to decompose formic acid. Pakes and Jollyman did not show, however, whether failure to effect this anaerobic decomposition of formic acid involves also an inability to bring about an aerobic breakdown to carbon dioxide and water.

THE OXIDATION OF FORMIC ACID.

In their studies on the dehydrogenating activity of bacteria Quastel and Whetham [1925] showed that in the presence of methylene blue formates are rapidly dehydrogenated by bacterial suspensions, the rate of reduction of methylene blue by formates under standard conditions being greater than that of any other substance tried with the exception of the sugars. When molecular oxygen replaces methylene blue as hydrogen acceptor the relatively high rate of oxidation of formate, as compared with that of other substances, is no longer shown, the velocity of its oxidation, in fact, not attaining that of lactate. Measurements of oxygen uptake show that in the case of *B. coli communis* the oxidation is complete, that is, proceeds according to the equation [Cook and Stephenson, 1928]

$$H.COOH + O = H_2O + CO_2.$$

Arising out of these observations, it is of interest to know whether the

anaerobic mechanism studied by Pakes and Jollyman, whereby certain bacterial species when growing on broth decompose formic acid according to the equation

$$H.COOH = H_2 + CO_2,$$

is identical with the aerobic mechanism studied by Cook and Stephenson, by which suspensions of dead bacteria in phosphate buffer decompose formates according to the equation

$$H.COOH + O = H_2O + CO_2.$$

The preparation of formic dehydrogenase.

The first step in the attack on this problem was an attempt to make a cell-free preparation of formic dehydrogenase in a manner analogous to that used in the preparation of lactic dehydrogenase from *B. coli* [Stephenson, 1928] and from yeast [Bernheim, 1928], the citric dehydrogenase from liver [Bernheim, 1928] and the succinic dehydrogenase from muscle [Ohlsson, 1921]. Autolysis of the cells in a buffer solution proving unsuitable for the purpose, the more drastic method of digesting them with trypsin was resorted to. A suspension of *B. coli* was prepared by growing the organism on plates about 6 in. in diameter containing tryptic broth agar to which 0·5 % sodium lactate had been added; the organisms were grown for 24 hours, washed off the plates with dilute phosphate buffer at p_H 6·0, centrifuged, and washed three times with Ringer's solution. The growth from 24 plates was suspended in about 100 cc. of Ringer's solution and aerated for half an hour to remove traces of oxidisable materials. The digestion was carried out by adding 5 cc. of Benger's *liquor pancreaticus* to 100 cc. of suspension, buffering the mixture at p_H 7·6, and incubating at 37°; it was found that sodium fluoride had no effect on the action of the digested preparation on formic acid, and 1 % sodium fluoride was therefore added to prevent growth of contaminating organisms.

At intervals during the incubation samples were withdrawn and tested to ascertain the reduction time of methylene blue by the three hydrogen donators formate, succinate and lactate. It was found that the activity of these three dehydrogenases was greatly increased by digestion with trypsin; the succinic and lactic dehydrogenases, however, soon disappeared (being very susceptible to the action of fluoride) but the formic dehydrogenase was stable and attained five times its original activity (Fig. 1). When this very active digested suspension was centrifuged and filtered through a glass filter (G 4), about 50–60 % of its activity was found in the filtrate; for instance, in one experiment the following reduction times were obtained:

	Reduction time mins.	secs.
Original suspension	25	20
Suspension digested for 4 weeks ...	5	40
Supernatant fluid after centrifuging	7	45
Residue	25	0
Filtrate through glass filter ...	10	0

The filtrate, examined under the microscope, was entirely free from whole cells, but contained large quantities of small particles of cell débris from which

the enzyme could not be separated; by repeated centrifuging at high speed a supernatant fluid was obtained which was almost inactive, while the small quantity of residue, made up to the same volume, possessed almost all the activity of the original filtrate.

	Reduction time mins.	secs.
Filtrate	3	10
Final supernatant fluid	36	0
Residue	3	20

Filtration through a Seitz filter and through a layer of kieselguhr gave similar results. The formic dehydrogenase was not therefore obtained in the same soluble state as Stephenson's lactic dehydrogenase, but a very active preparation free from whole cells was made.

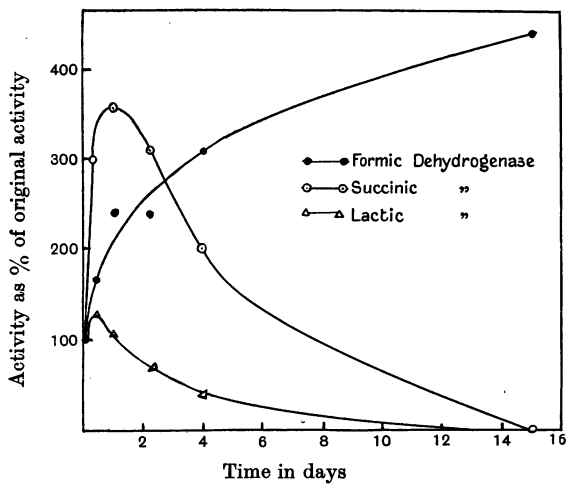

Fig. 1. Effect of tryptic digestion on the activity of the dehydrogenases of *B. coli*. (Reciprocals of reduction times.)

The *liquor pancreaticus* used in the digestion, besides containing trypsin, was rich in amylase, and it was thought advisable to determine which of these two enzymes was producing the effects described. By taking the *liquor pancreaticus* to p_H 3·0 by cautious addition of hydrochloric acid, and restoring it to neutrality after 10 minutes at room temperature, it was possible to destroy more than 99·5 % of the amylase, while leaving more than 70 % of the trypsin. Two digestions, carried out simultaneously with treated and untreated *liquor pancreaticus*, gave practically identical results with regard to the increase in activity of the formic dehydrogenase, so the result may be attributed entirely to the action of the trypsin.

It is clear from these experiments that the effect of tryptic digestion on a suspension of *B. coli* is to increase the activity of the formic dehydrogenase (as tested by the methylene blue technique), while eliminating the other dehydrogenating mechanisms of the cell. The next point was to ascertain whether the formic dehydrogenase preparation thus obtained was capable of

oxidising formic acid by means of molecular oxygen. This was done by measuring the oxygen uptake of the enzyme with formic acid in a Barcroft apparatus, the two cups containing:

Left-hand cup	Right-hand cup
1 cc. enzyme preparation	1 cc. enzyme preparation
1 cc. phosphate buffer, p_H 6·0	1 cc. phosphate buffer, p_H 6·0
1 cc. water	1 cc. formic acid (M/40 or M/100)

The results obtained were as follows (mm.^{3} O_2):

M/100 formic acid	M/40 formic acid
22	20
18	16
23	16
18	21
Theoretical = 112	Theoretical = 280

When a fresh suspension of *B. coli* was used, the oxygen uptake corresponded to complete oxidation, *e.g.*

with M/40 formic acid	291 and 294 mm.^{3} O_2	(theoretical = 280);
with M/20 formic acid	523	(theoretical = 560).

It has previously been shown by Stephenson that lactic acid is not oxidised aerobically by lactic dehydrogenase alone, but in the presence of an autoxidisable dye such as methylene blue is oxidised completely to pyruvic acid. Since the formic dehydrogenase preparation was so active in reducing methylene blue, it seemed probable that addition of the dye to the Barcroft cup would enable the system to use molecular oxygen to oxidise the formic acid, but in this case the oxygen uptake remained unaltered.

M/40 formic acid + 0·5 cc. 1/5000 methylene blue 32 and 19 mm.^{3} O_2.

It now remained to account for the small constant uptake, corresponding to oxidation of 0·0006 M formic acid. The amount, as is shown by the figures given above, was independent of the concentration of formic acid; it therefore appeared possible that on oxidation a substance was formed which, when its concentration reached a certain value, inhibited further enzyme action. Products of the reaction which might cause this inhibition were then sought for; oxalic acid, formaldehyde and peroxide were tried. The first two were found to have no effect, either on the activity of the formic dehydrogenase preparation (tested by the methylene blue technique) or on the oxidation of formic acid by a fresh suspension of *B. coli*, even when used in a concentration greater than could possibly be present in these experiments, viz. oxalic acid N/100, formaldehyde M/10. Peroxide could not be detected in the contents of the Barcroft cups by the titanium and guaiacum tests, whilst hydrogen peroxide in a concentration of 0·0003 %, which is easily detectable by these tests, caused no inhibition of the dehydrogenase. If these have no effect on the dehydrogenase it may be assumed that they do not affect the oxygen uptake in the presence of methylene blue. Bernheim [1928] attributed the inability of the soluble citric dehydrogenase to take up oxygen, even in the presence of methylene blue, to destruction of the enzyme by mere shaking in

air. This explanation cannot be applied in the case of the formic dehydrogenase, for samples which have been shaken alone in a Barcroft cup for 2 hours show only a small decrease in activity, while samples shaken in the presence of formic acid are completely inactivated, *e.g.*

		Reduction time mins.	secs.
Original preparation		2	50
After 2 hours' shaking without formic acid	(1)	6	25
	(2)	6	0
After 2 hours' shaking with formic acid ...	(1)	>60	0
	(2)	>60	0

The only method by which the formic dehydrogenase could be shown to oxidise completely a known quantity of formic acid was by causing it to reduce an equimolecular amount of methylene blue, and measuring the oxygen required for re-oxidation of the leuco-methylene blue. Barcroft apparatus were set up containing:

Left-hand cup	Right-hand cup
1 cc. enzyme preparation	1 cc. enzyme preparation
0·5 cc. buffer, p_H 6·0	0·5 cc. buffer, p_H 6·0
1 cc. *M*/20 methylene blue	1 cc. *M*/20 methylene blue
0·5 cc. water	0·5 cc. *M*/20 formic acid

To determine the time required for complete oxidation of the formic acid, a Thunberg tube was prepared with 1 cc. enzyme preparation, 0·5 cc. buffer, 0·5 cc. *M*/20 methylene blue, 0·5 cc. *M*/20 formic acid and 0·5 cc. water; the time taken for reduction of the methylene blue in this tube gave an approximation to the time required in the Barcroft experiment. The Barcroft apparatus was evacuated and placed in the bath at 40° for a rather longer time than that found by the above method, and then air saturated with water vapour at 40° was admitted to both cups. The oxygen uptake was then measured in the usual way, and was found to be of the right order for complete oxidation (Fig. 2, p. 1192):

0·5 cc. *M*/20 formic acid

	264 mm.^{3} O_2
	366
	284
Mean	305

Theoretical = 280

It thus appears that treatment of the cells with trypsin has increased the dehydrogenase activity, but decreased the capacity of the cell to oxidise formic acid by molecular oxygen.

The action of the formic dehydrogenase preparation in liberating molecular hydrogen from formates was next investigated. Experiments were made in test-tubes containing Durham tubes, formic acid (0·01–0·1 *M*), phosphate buffer (p_H 5·0–9·0), and the enzyme, these were incubated anaerobically. In no case was any detectable hydrogen liberated. It thus seems that tryptic digestion of *B. coli* suspensions, though increasing the activity of the dehydrogenase towards methylene blue, has resulted in the elimination of the mechanism liberating molecular hydrogen anaerobically.

Additional evidence as to the identity or non-identity of the aerobic and anaerobic mechanisms for the decomposition of formic acid was then sought for by the study of an organism in which the latter mechanism is known to be absent. For this purpose *B. typhosus* was selected.

Presence of formic dehydrogenase in B. typhosus.

A suspension of *B. typhosus* was prepared in the same way as in the case of *B. coli*. The reduction times with methylene blue were as follows:

	Reduction time (mins.)
Blank (no hydrogen donator)	100
Formic acid *M*/20	$5\frac{3}{4}$
Succinic acid *M*/20	17
Lactic acid *M*/20	$3\frac{1}{2}$

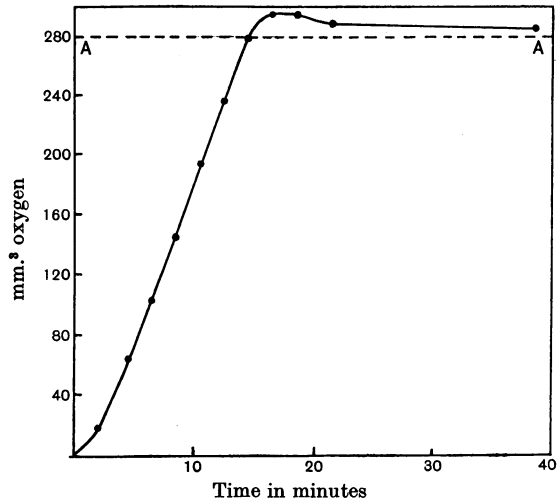

Fig. 2. Oxygen uptake of leuco-methylene blue produced by oxidation of formic acid by the dehydrogenase preparation. *A* represents the theoretical amount.

Hence *B. typhosus* shows a marked power of activating formic acid as a hydrogen donator to methylene blue. This organism was also sown into test-tubes containing Durham tubes with tryptic broth and 0·5 % formic acid; no gas was produced after 3 days' incubation, while similar tubes sown with *B. coli* produced much hydrogen in 24 hours. This result is in accordance with Pakes and Jollyman's findings for non-gas-producing organisms. Thus it appears that in the case of *B. typhosus* we have an example of an intact organism possessing the mechanism for dehydrogenating formates but lacking the mechanism for the anaerobic decomposition of formic acid into carbon dioxide and molecular hydrogen. In this respect the intact cell of *B. typhosus* resembles the digested cell of *B. coli*.

THE COURSE OF THE ANAEROBIC DECOMPOSITION OF FORMIC ACID.

Preliminary experiments showed that without strong buffering the reaction was stopped by the increasing alkalinity; phosphate in greater concentration

than $M/10$ also inhibited the reaction. Finally $M/10$ phosphate buffer at p_H 6·0 and $M/10$ formic acid were used, the p_H after complete decomposition of the formic acid being about 9.

The formic acid, buffer solution and suspension of washed and aerated cells of *B. coli* were put into a 250 cc. flask which was placed in a water-bath at 37° and connected to a wash-bottle to prevent leakage of oxygen into the flask. Oxygen was supplied through a T-piece placed between this wash-bottle and two drying tubes containing calcium chloride, which were followed by a tube containing a platinum spiral for the combustion of hydrogen, weighed calcium chloride tubes for the estimation of the water formed and baryta tubes for the estimation of carbon dioxide.

The flask was first washed out with a rapid current of oxygen-free nitrogen. Samples for the estimation of formic acid were withdrawn every 24 hours through a capillary tube, bent through 180° at the top and carrying a tap; these estimations were carried out by a method based on that described by Dakin [1913]. The sample (containing 5–10 mg. of formic acid) was diluted with 30 cc. of 50 % alcohol, acidified with phosphoric acid and distilled *in vacuo* into excess of ice-cooled sodium hydroxide; the residue was redistilled with a further 20 cc. of 50 % alcohol. The combined distillates were evaporated to about 10 cc., placed in a pressure bottle, slightly acidified with acetic acid, and heated with a twenty-fold excess of saturated mercuric chloride solution for 7 to 8 hours in a boiling water-bath, the precipitated calomel being collected and weighed. Each estimation was carried out in duplicate, these agreeing to within 1–2 %. Known quantities of formic acid gave yields of only 98–99 % of the theoretical amount of calomel.

The amounts of hydrogen and carbon dioxide formed during each period of 24 hours were estimated by blowing 2 litres of oxygen-free nitrogen through the apparatus in the course of about 2 hours. With an apparatus of the size used, this would mean that, assuming complete mixing of the gases, 99·98 % of the gas in the flask had been washed over. The hydrogen was measured by adding an excess of oxygen, drying the mixture, passing it over the heated platinum spiral and weighing the water collected in the calcium chloride tubes. The carbon dioxide was estimated in the residual gas by passing it through the series of bottles containing standard baryta and titrating the remaining baryta. The apparatus was tested by passing moist air through the series of tubes; no increase in the weight of the calcium chloride tubes was found. Two litres of air containing 1·84 % of hydrogen (previously estimated on the Haldane apparatus) gave 0·0310 g. of water, corresponding to a yield of 104 %.

Results.

After a preliminary period of about 24 hours, during which very little decomposition took place, the reaction proceeded at a uniform rate for several days, and then decreased in velocity (Fig. 3). A possible interpretation of the shape of the curve will be discussed later; these results serve to show, however,

that at every stage the quantities of hydrogen and carbon dioxide formed correspond to the amount of formic acid decomposed, according to the equation

$$H.COOH = H_2 + CO_2.$$

THE PRODUCTION OF HYDROGEN IN AEROBIC CONDITIONS.

In Cook and Stephenson's work on the oxidation of formic acid by *B. coli* the amount of oxygen taken up in a Barcroft apparatus was exactly that required by the equation

$$H.COOH + O = H_2O + CO_2;$$

if any hydrogen had been produced their results would have been too low. In order to find out whether in aerobic conditions any hydrogen can be formed Barcroft apparatus were set up containing:

Left-hand cup	Right-hand cup
1 cc. suspension of *B. coli*	1 cc. suspension of *B. coli*
0·5 cc. buffer, p_H 6·0	0·5 cc. buffer, p_H 6·0
1 cc. water	1 cc. 0·4 *M* formic acid
0·5 cc. tryptic broth	0·5 cc. tryptic broth

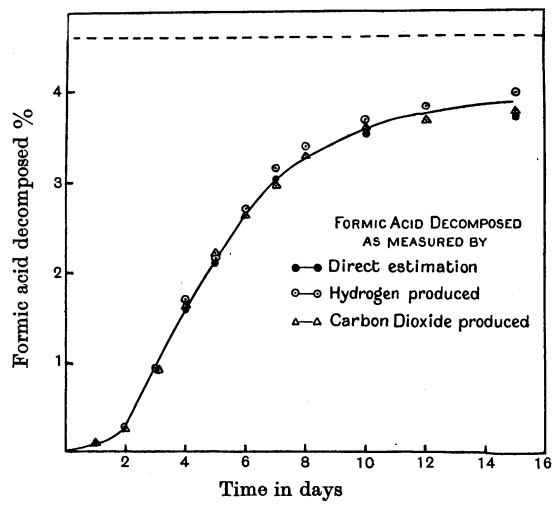

Fig. 3. The course of the anaerobic decomposition of formic acid.

The tryptic broth was added because the production of hydrogen is always more rapid in the presence of broth. After the apparent oxygen uptake had reached 2750 mm.^{3}, corresponding to oxidation of about 60 % of the formic acid, the rate of oxidation slowed down, and at this point 10 cc. of the gas in the right-hand cup were transferred to a Haldane apparatus. Analysis of the gas showed that it contained no detectable amount of hydrogen (*i.e.* less than 0·02 %), demonstrating that less than 0·15 % of the total formic acid (taking the volume of the Barcroft cup as roughly 40 cc.) had been decomposed according to the equation

$$H.COOH = H_2 + CO_2.$$

A state equivalent to the aerobic condition can be produced by adding

various hydrogen acceptors other than oxygen to the medium, *e.g.* nitrate and fumarate. Experiments with these substances were carried out in test-tubes with Durham tubes; each tube contained 0·1 *M* formic acid, 0·1 *M* phosphate buffer at p_H 6·0, tryptic broth and 0·1 *M* potassium nitrate or sodium fumarate, and all were sown from a fresh culture of *B. coli*. In the absence of a hydrogen acceptor growth was good and large amounts of gas were produced; in the other tubes the growth was equally good, but in the presence of nitrate no gas was produced at all, and in the presence of fumarate a very small amount of gas was usually observed.

It may therefore be concluded that in thoroughly aerobic conditions the decomposition of formic acid into hydrogen and carbon dioxide is entirely suppressed, its place being taken by the oxidising reaction.

Discussion.

The obvious explanation of the mechanism of the decomposition of formic acid is that the primary reaction is the splitting of the molecule into hydrogen and carbon dioxide; the hydrogen is then, in the absence of a hydrogen acceptor, liberated in the molecular state, or, in the presence of a hydrogen acceptor such as oxygen, nitrate or methylene blue, oxidised to water or leuco-methylene blue. It is found, however, that *B. coli* cells, after digestion with trypsin, although still capable of actively dehydrogenating formic acid, can no longer decompose it into hydrogen and carbon dioxide; similarly *B. typhosus* can dehydrogenate formic acid, but cannot liberate hydrogen anaerobically. This leads one to suppose that in *B. coli* there are two distinct mechanisms for the decomposition of formic acid, one anaerobic and the other aerobic, of which only the latter is present in *B. typhosus*. If this were so, one would expect to find that, even in thoroughly aerobic conditions, a part of the formic acid (provided that its concentration is well above that required for saturation of the dehydrogenase) will be decomposed into hydrogen and carbon dioxide. Experiment shows that, as far as can be detected by the method employed, thorough aeration completely suppresses the formation of hydrogen, showing that there is after all some connection between the two processes. One finally reaches the conclusion that the process common to both reactions is the activation of the hydrogen atoms of the formic acid by the dehydrogenase, and that for the union of the two atoms to form molecular hydrogen a further enzyme mechanism is necessary, which is found only in certain organisms, and which does not operate in the presence of hydrogen acceptors.

The relation of dehydrogenation and of hydrogen production to the viability of the organisms.

It has been shown previously [Cook and Stephenson, 1928] that the bacterial oxidation of lactate and formate is independent of the number of living cells present. Since an experiment on the production of hydrogen lasts at least a week, so that the surviving cells have ample opportunity of growing

on the products of autolysis of the dead ones, it was advisable to ascertain whether the production of hydrogen was dependent on the viability by following the change in the number of viable cells during the course of an experiment. The counting was carried out by the method described by Wilson [1922], and the formation of hydrogen was followed in Durham tubes containing buffer at p_H 6·0, 0·5 % formate and the suspension of *B. coli*. This method could be made roughly quantitative by simply measuring the length of tube occupied by the gas, and by taking the mean of six similar tubes curves were obtained of the same shape as those given by the more accurate method already described. The gas in the Durham tubes was in a number of cases analysed in the Haldane apparatus and was found to be of very nearly constant composition, viz. 20 % carbon dioxide and 80 % hydrogen.

The results showed that when a suspension of *B. coli* is incubated with formate and buffer, the viable count decreases rapidly during the first 24–48 hours, while no gas is given off, and during the subsequent evolution of hydrogen remains practically constant, either slightly decreasing (Fig. 4) or slightly

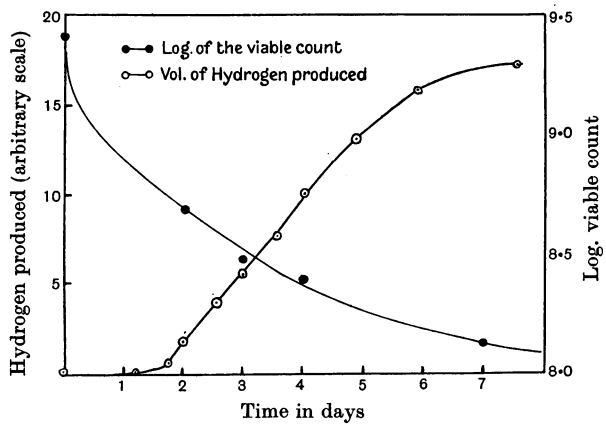

Fig. 4. Changes in the viable count of a suspension of *B. coli* during the decomposition of formic acid into hydrogen and carbon dioxide.

increasing (Fig. 5). If the suspension is first treated with ultra-violet light till the viable count is reduced to about one-millionth of its original value, then on incubation with formate the cells multiply steadily until, after about 4 days, the count is roughly equal to that attained at the end of the experiments with untreated cells; at this point the gas production begins and the count remains almost stationary (Fig. 6). These results suggest that the decomposition of formic acid into hydrogen and carbon dioxide depends not merely on the number of viable cells, but on the rate of their multiplication, for in each case the hydrogen is given off when the rate of multiplication is at its maximum, that is, when the death-rate is balanced by the division of the survivors.

Two other pieces of evidence seem to support this view; the first is that in the presence of tryptic broth, where the cells can multiply very rapidly, the

decomposition of formic acid is very much more rapid than in the absence of broth. The second is that when various concentrations of certain growth-inhibiting substances were added to the solution, a smaller concentration was required to prevent hydrogen production by a suspension of *B. coli* than was required to prevent growth of the organism in broth, *e.g.*

	%	Growth	Hydrogen
$FeCl_3$	0·032	+ +	+
	0·064	+	+
	0·128	−	−
$ZnSO_4 7H_2O$	0·12	+ +	+
	0·18	+ +	+
	0·27	−	−
$NiSO_4 7H_2O$	0·045	+ +	+
	0·068	+ +	−
	0·101	−	−
NaF	0·1	+ +	+
	0·24	+ +	−
	0·53	+	−

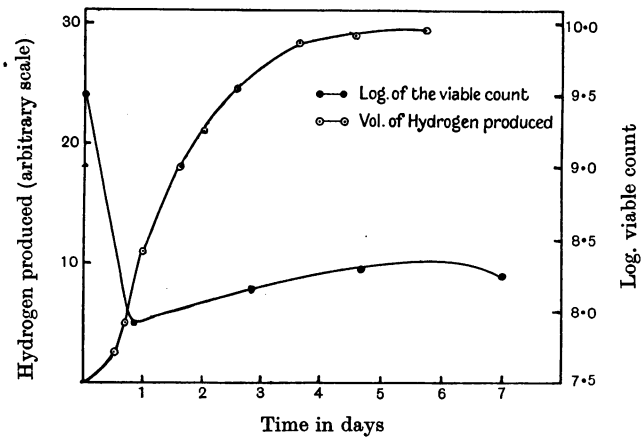

Fig. 5. Changes in the viable count of a suspension of *B. coli* during the decomposition of formic acid into hydrogen and carbon dioxide.

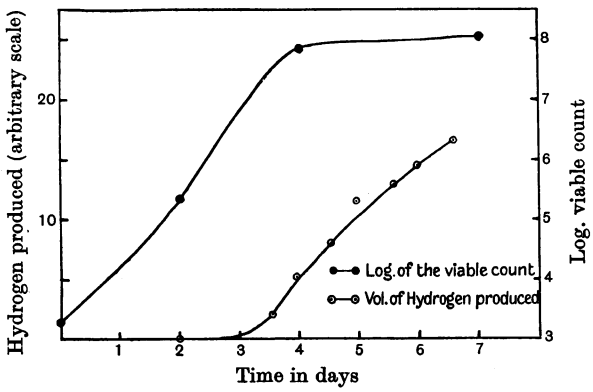

Fig. 6. Changes in the viable count of a suspension of *B. coli* that had been treated with ultra-violet light, during the decomposition of formic acid into hydrogen and carbon dioxide.

All that can be said definitely on this subject at present is that no hydrogen has been obtained in conditions in which growth of the organism was prevented, and that whenever hydrogen was produced multiplication of the organisms could be demonstrated.

SUMMARY.

1. Tryptic digestion of a suspension of *B. coli* cells causes a transitory increase in the activity of the succinic and lactic dehydrogenases, and a permanent one in that of the formic dehydrogenase to five times its original value. From the product of the digestion a preparation of formic dehydrogenase can be made, which is free from intact cells but contains cell débris.

2. This preparation will not oxidise formic acid by means of molecular oxygen, nor will it decompose it into hydrogen and carbon dioxide anaerobically.

3. In thoroughly aerobic conditions *B. coli* oxidises formic acid completely, giving no free hydrogen.

4. *B. typhosus* reduces methylene blue in the presence of formate, but cannot liberate molecular hydrogen from formate.

5. The bearing of these facts on the relation between the two modes of decomposition of formic acid is discussed.

I wish to thank Sir F. G. Hopkins for his encouragement and Miss M. Stephenson for constant help and advice while this work was being done.

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