DESCRIPTION OF PLATES.

1. Three days' growth in fresh homogenous plasma.
2. Three days' growth in homogenous plasma three days old.
3. Three days' growth in homogenous plasma ten days old.
4. Three days' growth in fresh autogenous plasma.
5. Five days' growth in fresh autogenous plasma.
6. Five days' growth in fresh homogenous plasma.

The Decomposition of Formates by Bacillus coli communis.

By Egerton Charles Grey, 1851 Exhibition Scholar.

(Communicated by Dr. A. Harden, F.R.S. Received February 19,—Read March 26, 1914.)

(From the Biochemical Department of the Lister Institute.)

Many observations have been made on the variability of gas production by intestinal bacteria under natural conditions (see Penfold (1911) and Arkwright (1913), where literature is quoted).

Penfold has found that by artificial selection of *Bacillus coli communis* in the presence of sodium chloroacetate, strains may be isolated which produce no gas from glucose and gas in lessened amount from mannitol, although in both cases acid is produced as with the normal organism. The writer has also shown that by artificial selection of *B. coli communis* by the chloroacetate method of Penfold, various stages between the original gas-producing and the selected non-gas-producing strain may be obtained, and the changes have been found to be associated in part with the disappearance of the enzyme which decomposes formic acid (1914). It was found that two kinds of artificially selected strains could be produced from the original strain of *B. coli communis*; one unable to decompose formic acid, and the other still able to bring about this decomposition provided glucose were present. The artificially selected organism, which could not decompose formates even in the presence of glucose, was likewise unable to produce gas from mannitol, whereas the organism which still retained the power of decomposing formates was also able to produce gas from mannitol, although it produced this gas in an amount approximately equal to one-half of that produced under the same conditions by the original *B. coli communis* from which it was derived. It seemed, therefore, likely that by a closer study of

the manner in which formic acid is decomposed by the natural and artificially selected varieties of intestinal bacilli it might be possible to gain information concerning the mechanism of the change brought about in the organism by growth on chloroacetate agar which leads to the selection of strains in some cases unable to decompose formic acid and in other cases unable to produce it to the same extent as the normal strains from which they have been derived.

It seemed also of importance to determine what use the decomposition of formic acid might be to the organism. Pakes and Jollyman (1901) and Harden (1901) have shown that *B. coli communis* is capable of decomposing a considerable amount of sodium formate, and that if a small quantity of glucose be added, the amount of hydrogen produced over and above that which could be derived from the glucose added is far greater than the amount produced in the absence of the sugar.

The writer has employed an artificially selected strain of *B. coli communis* obtained by the chloroacetate method; this strain produced in three days no gas from sodium formate peptone water, and only acid but no gas from glucose peptone water, but produced from a mixture of the two sufficient gas to fill the Durham gas tube (length 45 mm.) in 24 hours. The non-production of gas from sodium formate peptone water alone is due, not to the inability of the organism to decompose formic acid, but to the inhibitory action of the alkali due to the natural alkalinity of sodium formate; for if the sodium formate peptone water were acidified with sulphuric acid until the solution imparted a pink colour to litmus, it was found that a small quantity of gas was produced by growth of the artificially selected organism therein for two or three days.

Other sugars and polyhydric alcohols have been employed with similar results, which are discussed under Table II.

By a quantitative study of the decomposition by the bacillus in question of a mixture of glucose and calcium formate, the writer has been able to show that both the amount of glucose and that of formate decomposed is increased (Table III), and there can be little doubt that the formate and sugar are mutually helpful, in that the alkali produced by the decomposition of the former and the acid produced from the latter by neutralising one another maintain that approximately neutral condition of the medium which, as has been proved, is most favourable for the action of the organism.

Experimental.

The Examination of the Behaviour of Non-gas-producing Organisms towards Formates as a means of Deciding whether the Organism has been Derived from an Original Gas-producing Strain.

It has been mentioned above that by artificial selection of *B. coli communis* it is possible to obtain strains which do not produce gas from glucose, and that this phenomenon consists in part, in some cases, in a lessened power to decompose formic acid possessed by the selected organism. In the case of the strains examined by Penfold and Harden (1912) the power of decomposing formic acid was in all cases retained by the selected strains, and certain strains examined in the course of this work were found likewise to have retained this power. In the case of one strain, however, the power to decompose formic acid had been entirely lost. It may, therefore, be considered as probable that the strain incapable of decomposing formic acid represents a more advanced stage in the process of selection, and that, therefore, this type would be more permanent in character. Such indeed has proved to be the case, for while the strain which retains the power to decompose formic acid tends to revert in its properties to the parent organism as regards the production of gas from glucose, the other strain, which cannot decompose formic acid, shows no such tendency, although it has been frequently sub-cultured during the course of seven months.

In view of the fact that the more permanent non-gas-producing type of artificially selected strain is unable to decompose formic acid, it may be suggested that the same phenomenon might be exhibited by naturally occurring non-gas-producing organisms, and that in order to decide whether a strain which, at any particular time, does not produce gas has been recently derived from a gas-producing strain, an examination of its behaviour towards formic acid might be of crucial importance.

It frequently happens that organisms isolated from natural sources differ apparently only as regards the power to produce gas from carbohydrates and allied substances, and the question arises as to whether the one organism may have recently been derived from the other. Arkwright (1913), for example, has obtained varieties of *B. acidi lactici* differing in the aforesaid respect, both strains occurring in the same sample of urine, and he was also able to show that in certain cases the non-gas-producing strain could be trained to decompose sodium formate if grown for some time on a peptone water medium containing this salt. The writer has found that the power to produce gas from mannitol may, in some instances, be made to disappear by

simply allowing a broth culture of *B. coli communis* to remain unchanged for three months, or by growth of the gas-producing organism anaërobically in peptone solution containing mannitol in the presence of chalk for about one month. At the end of the period described, if a loopful of the culture be plated out on to agar, many of the colonies which grow at 37° will be found to produce no gas when inoculated into mannitol peptone water tubes. This change may be seen from Table I.

Table I.—The Disappearance from *B. coli communis* of the Power to Produce Gas from Mannitol by Continuous Growth of the Normal Organism in Unchanged Cultures.

History of the culture.	Production of gas.	
	Mannitol.	Glucose.
Normal *B. coli* recently isolated, average 46 normal strains	30 mm. gas	21·0 mm. gas.
The above-mentioned normal strains after being kept in unchanged broth 4 months, average 6 strains	25 ,,	22·0 ,,
Kept in unchanged broth 4 months, average 12 strains	12 ,,	20·0 ,,
,, ,, 4 ,, ,, 12 ,,	5 ,,	18·5 ,,
,, ,, 4 ,, ,, 8 ,,	2 ,,	20·5 ,,
,, ,, 4 ,, ,, 9 ,,	Nil	21·0 ,,

The strains described in Table I, which did not produce gas from mannitol, were examined after growth on broth during several sub-cultures and were found not to produce gas from mannitol when inoculated from the broth tubes into mannitol peptone water. Thus the acquired character is inherited for a considerable time under these conditions. It will be seen from the foregoing table that no change has been brought about in the power to produce gas from glucose, and this is also true for dulcitol. Nevertheless, if by simple growth in peptone water *B. coli communis* yields a strain incapable of producing gas from mannitol, it would seem not unlikely that some similar process might, with time, lead to the disappearance of the power to produce gas from glucose, but such has not so far been observed.

In deciding whether an organism possesses the formic acid decomposing enzyme, which it is suggested here should be used as a criterion of a gas-producing organism, it is not convenient or sufficient to observe whether gas is produced from peptone water containing sodium formate. The test should be made with a mixture of sodium formate and glucose in such proportions that the sodium carbonate which will result from the decomposition of the formate will be approximately sufficient to neutralise the acid which will

be produced from the carbohydrate. A convenient mixture is 1·5 per cent. carbohydrate and 0·5 per cent. sodium formate in 1 per cent. peptone water. It will be found under these circumstances that whereas an organism may give only a few bubbles, or even no gas at all, from sodium formate peptone water alone, and none at all from glucose peptone water alone, the mixture may yield gas with great rapidity, so that in 20 hours a Durham tube may be completely filled. This increased gas production is due chiefly to the decomposition of the formate, but partly also to gas which may be produced from the sugar when the solution is maintained neutral, as will be described later.

This increased gas production from formates in the presence of carbohydrates is strikingly illustrated in the case of a selected strain of *B. coli communis* obtained by the chloroacetate method, as will be seen from the following table. The numbers represent millimetres of the tube occupied by gas in the Durham tubes of 45 mm. length.

Table II.—The Effect of Addition of Carbohydrates and Allied Substances on the Decomposition of Sodium Formate by an Artificially Selected Strain of *B. coli communis* producing only a Minute Quantity of Gas from Glucose.

Time.	Sodium formate.	Glucose.	Lactose.	Mannitol.	Dulcitol.	Sorbitol.	Glycerine.
			(Concentration of the Sugar or Alcohol 2 per cent.)				
hours.							
12	Nil	Nil	Nil	Trace	Nil	Trace	Nil
24	,,	,,	,,	11	,,	12	,,
36	,,	Minute bubble	Minute bubble	23	,,	25	,,
60	,,	No increase	0·5	No increase	,,	No increase	Trace
84	,,	,,	2·0	,,	3	,,	1
108	,,	,,	3·0	,,	30	,,	1
132	,,	,,	3·0	,,	37	,,	1
			Evolution of Gas from the above Carbohydrates and Alcohols after Admixture with Sodium Formate. (Carbohydrate or Alcohol 1·5 per cent., Sodium Formate 0·5 per cent.)				
12		12	4·0	Trace	Nil	2	Nil
24		Full	Full	10	,,	10	,,
36				34	,,	Full	,,
60				Full	,,		5
84					5		7

The following facts should be noted in connection with the experiment described above :—

(1) The non-production of gas from formate peptone water alone was due, in part, to the natural alkalinity of the medium. To demonstrate this varying quantities of N/10 H_2SO_4 were added to a series of sodium formate peptone water tubes, which were then inoculated with a loopful of a broth culture of *B. coli communis*. It was found that in those tubes in which the reaction to litmus was nearest to neutral, there was a slight production of gas, whereas those which were distinctly alkaline or acid showed no gas at all.

(2) The manner in which the inoculation is made is also of importance. Several tubes of sodium formate peptone water were inoculated each with a loopful of a broth culture of *B. coli*, and another set of tubes were inoculated each with a loopful of an agar growth of the same organism. The former set of tubes produced no gas, the latter produced one-tenth of a Durham tube. This difference in the production of gas cannot be due simply to the size of the inoculation, for even when kept for 10 days the formate tubes inoculated from the original broth culture showed no production of gas. Probably, therefore, the bacillus when grown on agar contains more of the formic acid decomposing ferment than when grown in broth.

(3) The decomposition of sodium formate is not assisted in the same degree by mannitol as it is by glucose and the other sugars or by sorbitol, and it may be possible that this phenomenon is related to the fact already mentioned, that the power to produce gas from mannitol disappears from old broth cultures of *B. coli communis*, when these have remained unchanged for some months, and still more readily when the fluid contains mannitol and the products therefrom.

It should be noted also that, since less acid is produced from a hexahydric alcohol than from the same weight of a hexose when fermented by *B. coli communis*, the fact that the alcohol does not assist so well in the acceleration of the decomposition of the formate by the organism is in harmony with the view that it is the neutralisation of the medium by the acid produced by the carbohydrate or allied substance which is of assistance for the further decomposition of the formate.

The fact that in any particular experiment no gas may be produced from glucose peptone water is not a complete proof that an organism cannot produce gas at all from glucose, for the acid produced under circumstances in which no precaution is taken to neutralise the medium inhibits the decomposition of formic acid.

Quantitative Study of the Rate and Extent of Decomposition of Sodium Formate and Glucose by an Artificially Selected Non-gas-producing Strain of B. coli communis *when grown on them either separately or together.*

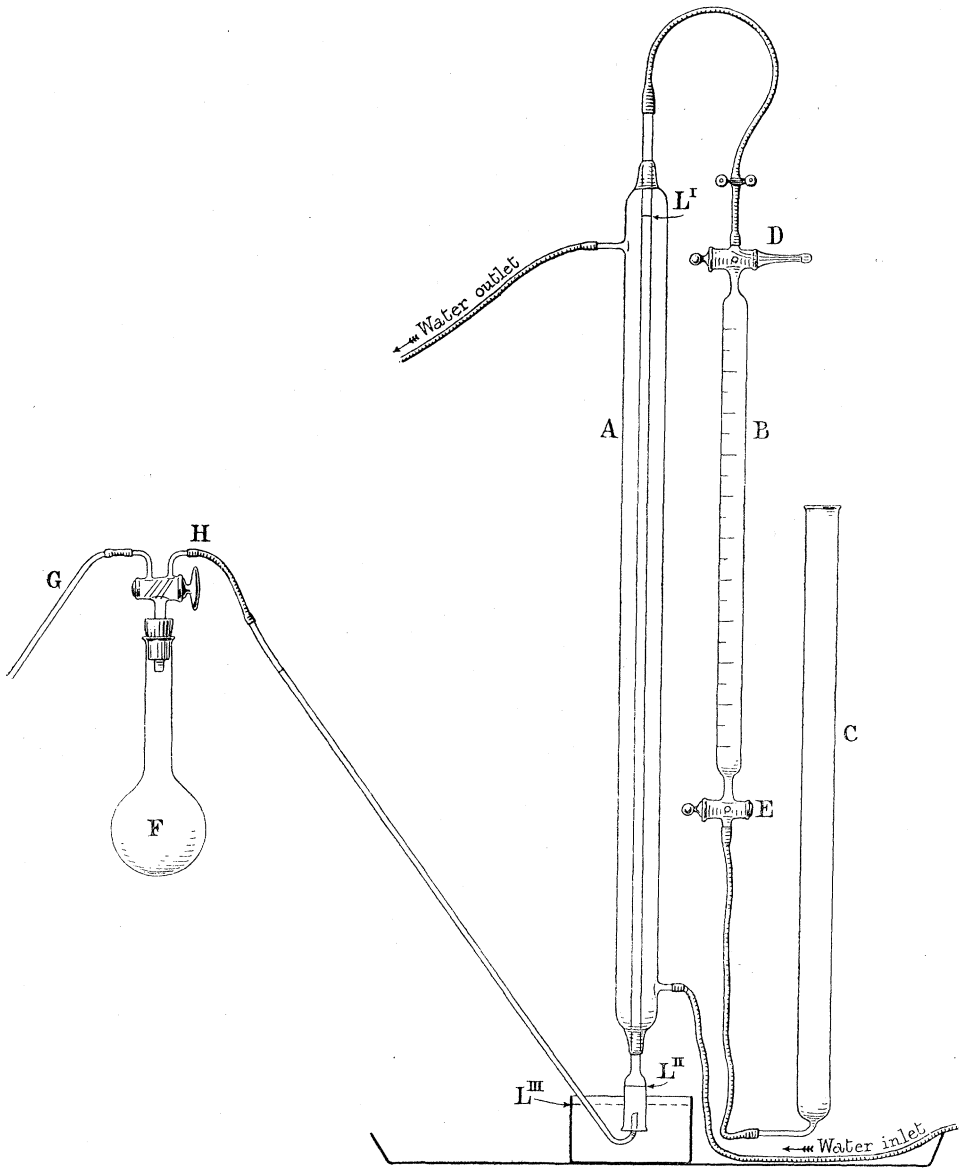
In order to determine the causes of the greatly increased gas production observed when *B. coli communis* was grown on a mixture of sodium formate and glucose, the change was followed quantitatively. For this purpose it was necessary to determine the weight of formic acid and glucose consumed in the reaction and the total carbon dioxide and acid produced, and also to measure the gas production from time to time. The method which was employed would be suitable for the examination of the decomposition of many other substances by bacteria, and it is therefore described in detail.

A quantity of 50 or 100 c.c. of 2 per cent. glucose in 1 per cent. peptone water is sterilised and inoculated with the organism. The cotton-wool plug, which should fit loosely, is pushed half-way down the neck of the flask, and the flask is connected with a Schiff's gas burette by means of a rubber stopper provided with a two-way tap. The burette, which is filled with mercury, is in connection with a reservoir for adjusting the pressure, as in the apparatus described by Harden, Thompson, and Young (1910). Before beginning the experiment, air may be removed from the flask by putting it in connection with the burette. On lowering the reservoir air passes into the burette. Nitrogen is then admitted to the flask by reversing the tap, and this process is repeated four or five times, when the oxygen will have been practically all removed. The flask is well immersed in a water-bath maintained at 37°. When it is desired to stop the reaction, the flask is removed from the water, and the contents are, after turning the two-way tap so as to put the flask in connection with the apparatus described below, carefully brought to the boil, the gas driven out displacing the mercury from the inner tube A (see figure).

Details of the Use of the Gas Collecting Apparatus.—The object of the apparatus is to collect all the gases which remain in the fermentation flask both free above the surface of the medium and dissolved in the fluid.

A is an ordinary Liebig's condenser set vertically and connected by a three-way tap D with a gas burette B accurately graduated. By putting D in connection with the pump or by raising the tube C, which must be filled with mercury, the mercury rises to fill B; the tap E is then closed. The tap D may now be reversed and mercury drawn up into the inner tube A from the reservoir L''' to the level L'. A circulation of water in the Liebig's condenser is not necessary for the condensation of the steam, but helps in keeping the temperature of the collected gas constant. To collect the gases

the flask F is heated carefully and the contents brought to the boil; the gas displaces the mercury from the inner tube A, and should the gas evolved be more than sufficient to fill A the tap D may be turned so as to connect



A and B, and the tap E turned so as to connect B and C, while C is lowered; the mercury in B falls with a corresponding rise of mercury in A.

The volume of the inner tube from a definite etched mark L″ to the tap

D, including the volume of the pressure tubing connecting A and B, having been previously determined, the total volume of evolved gases may be measured by raising the reservoir C, E being open, and D turned to connect A and B; the mercury then rises in B and falls in A, in which it is allowed to fall to the level L″. To correct for pressure an allowance may be made for the height of the mercury from the surface of the reservoir L‴ to L″, but it is also quite convenient to lower the whole Liebig's condenser until L″ coincides with L‴. The volume of gases in the graduated tube B is then observed, and this volume added to that of the inner tube A. A sample of the gases may now be conveniently removed by lowering C. When B contains sufficient of the gases for analysis, the whole apparatus B–C may, if desired, be removed from its connection with A.

The apparatus has been described in detail because it is of use for the determination of all gases remaining in the fermentation flask. In the experiments recorded in the present communication, however, it was only of value to determine residual carbon dioxide.

Details of the Determinations.—The carbon dioxide boiled off from the solution, as described above, is measured in the usual way. The flask is now detached from the apparatus and the contents filtered from the deposit of chalk, and the filtrate and washings precipitated in hot solution with ammonium oxalate. The precipitate of calcium oxalate is used to estimate the calcium corresponding to the total acids produced during the fermentation, an allowance being made for the calcium in the peptone. The filtrate from the calcium oxalate is acidified with oxalic acid and distilled in steam, the distillate neutralised with deci-normal potash and evaporated to dryness; the residue is dissolved in about 50 c.c. of water, and the formic acid determined by the reduction of mercuric chloride. The residue from the steam distillation is made up to a definite volume, and an aliquot portion used for the determination of the residual sugar by Bertrand's method after the removal of peptone by Patein's mercuric nitrate reagent.

The results of the experiment are summarised in Table III.

It will be seen from Table III that about ten times as much gas was produced by the selected strain of *B. coli communis* from calcium formate in the presence of glucose as was produced by it from calcium formate alone. The amount of sugar decomposed in the presence of calcium formate is considerably greater than in its absence, even when the medium is kept as far as possible neutral by chalk.

Table III.—Comparison of the Action of an Artificially Selected Strain of *B. coli communis* (Escherich) on Glucose alone; Glucose + Calcium Formate; Calcium Formate alone.

	Conditions of the experiment.			
	Glucose alone. Medium not neutralised during fermentation.	Glucose alone. Medium kept neutral by chalk.	Glucose + calcium formate + chalk.	Calcium formate alone.
Duration	99 hours	99 hours	120 hours	120 hours
Glucose before ...	3·385	1·6926	1·6926	None
„ after	3·2276	1·0628	None	„
„ consumed	0·1574	0·6098	1·6926	—
Formic acid before	None	None	0·5244	0·5244
„ „ after	0·0874	0·0249	0·0276	0·4988
„ „ consumed	—	—	0·4968	0·0256
CO_2 total gas ,.....	42 c.c.	96 c.c.	291 c.c.	12 c.c.
CO_2 from acids on chalk	41 „	90 „	161 „	—
CO_2 from formate	—	—	119 „	12 c.c.
CO_2 from sugar ...	—	6 c.c.	11 „	—

The medium contained in all cases 1 grm. peptone (Witte) in 100 c.c.

The actual carbon dioxide produced by the organism from calcium formate is in reality twice that actually evolved, for in the decomposition

$$(HCOO)_2Ca + H_2O = CaCO_3 + CO_2 + 2H_2$$

it is clear that one-half of the CO_2 is retained in combination with the calcium.

These results bring out, therefore, very clearly one object which is attained by the decomposition of formates by these bacteria, viz.: that the organisms are thereby supplied with the best possible neutralising agent. For the formate by being decomposed into carbon dioxide and hydrogen virtually liberates alkali within the bacterial cytoplasm, and thus not only neutralises the medium, but also the bacteria themselves. Moreover the calcium formate being itself neutral possesses none of the disadvantages which would arise from the presence of even a slight excess of alkali. It would be difficult to devise a more efficient means for maintaining neutrality in this case. I would suggest the utilisation of sodium or calcium formate as a neutralising agent in working with those organisms capable of decomposing it, especially for solid media, with which the addition of dissolved alkali from time to time would be impracticable.

Summary and Conclusion.

(1) The power of *B. coli communis* to decompose formic acid varies considerably when the organism has been kept for some time on artificial media.

(2) The decomposition of formates is inhibited by a very small excess of either acid or alkali and, therefore, a greatly increased decomposition of formates results if glucose is added, since the acid produced from the sugar neutralises the alkali from the formate.

(3) A method and apparatus are described by which the decomposition of various substances by micro-organisms may be followed quantitatively requiring only 50–100 c.c. of the solution.

(4) It has been suggested that in place of a solution of sodium formate a mixture of sodium formate 0·5 per cent. and glucose 1·5 per cent. should be used as a test of a gas-producing strain, since by this means the production of gas from formate is greatly increased, and it is also suggested that the test could be used as a criterion as to whether an organism, which has been recently isolated from some natural source and produces no gas from glucose peptone water, may be regarded as having been recently derived from a gas-producing strain.

(5) It has been shown that formates may be conveniently used as neutralising agents, and that thereby the activity of gas-forming organisms may be considerably increased.

In conclusion I would express my thanks to Prof. Harden, F.R.S., for help and criticism.

REFERENCES.

Arkwright, J. A., 'Journ. Hyg.,' vol. 13, p. 68 (1913).
Grey, E. C., this vol., p. 472 (1914).
Harden, A., 'Chem. Soc. Journ.,' vol. 79, p. 610 (1901).
Harden, Thompson and Young, 'Biochem. Journ.,' vol. 5, p. 230 (1910).
Harden and Penfold, W. J., 'Proc. Roy. Soc.,' B, vol. 85, p. 416 (1912).
Penfold, W. J., 'Roy. Soc. Med. Proc.,' p. 97 (1911).
Pakes and Jollyman, 'Chem. Soc. Journ.,' vol. 79, p. 386 (1901).