CCXCI. THE FORMATION OF HYDROGEN FROM GLUCOSE AND FORMIC ACID BY THE SO-CALLED "RESTING" *B. COLI.* II.

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In the first communication on this subject [Tasman and Pot, 1935] a critical examination was made of the relevant publications of Stickland [1929], Stephenson and Stickland [1931; 1932; 1933] and Yudkin [1932], and this examination was supported partly by theoretical considerations and partly by experimental data. As regards the latter, briefly, Tasman and Pot did not succeed in preparing suspensions of *B. coli* by the method employed by the English investigators, which would liberate gas in the anaerobic fermentation of glucose but not from sodium formate. If the bacterial suspension in question was prepared by previous cultivation in or on caseinogen-peptone, the formation of gas (H_2+CO_2) from both glucose and sodium formate was parallel.

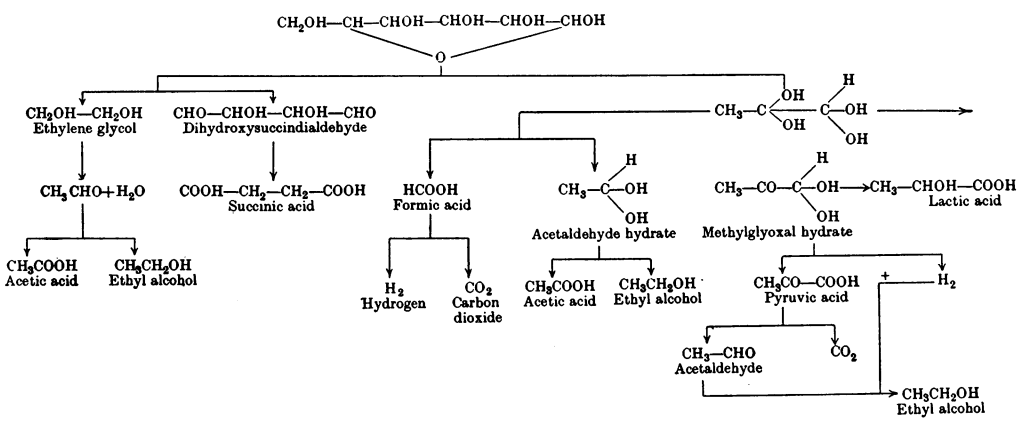
We also concluded that the hydrogen formed in the fermentation of glucose originated, in the majority of cases, from formic acid produced as an intermediate in the decomposition of this sugar.

As a continuation of these qualitative tests, experiments are described in this communication in which the fermentation of glucose, especially as regards the formation of hydrogen and carbon dioxide, has been investigated quantitatively.

Kluyver [1931; 1935] and his co-workers, amongst whom Scheffer [1928] warrants particular mention, have drawn up a general reaction scheme for the fermentation of glucose by microorganisms of the coli-typhosus-dysentericus group, which is reproduced below in abbreviated form.

Synopsis of the reactions taking place in the fermentation of glucose by B. coli.

(Abbreviated according to Scheffer.)



A discussion of the arguments which have led up to this reaction scheme will be omitted here; for this reference may be made to the publications of the authors mentioned above. Further discussion will be limited to a few practical consequences.

Methylglyoxal hydrate, which occupies a central position in this scheme, may decompose further in three different ways, namely, by:

(*a*) isomerisation to lactic acid;
(*b*) decomposition to pyruvic acid and hydrogen;
(*c*) fission into formic acid and acetaldehyde hydrate.

The pyruvic acid mentioned under (*b*) will break down further into acetaldehyde and carbon dioxide, after which this acetaldehyde will be reduced to ethyl alcohol by the hydrogen liberated in the formation of pyruvic acid. In all the cases we have investigated the fermented substrates have given a negative Voges-Proskauer reaction, so that the formation of acetylmethylcarbinol and 2:3-butyleneglycol may be left out of consideration. The decomposition of glucose represented under (*b*) is usually termed the "pyruvic acid scheme".

If fission of methylglyoxal hydrate takes place *via* formic acid (by the so-called "formic acid scheme"), a more or less considerable proportion of the latter will be decomposed into equivalent amounts of hydrogen and carbon dioxide, whilst the acetaldehyde produced along with the formic acid will be converted into equivalent amounts of acetic acid and ethyl alcohol.

If as in alcoholic fermentation the decomposition of glucose more or less follows the "pyruvic acid scheme", neither hydrogen and carbon dioxide nor acetic acid and ethyl alcohol will be formed in equivalent quantities, but the ratio of hydrogen to carbon dioxide must be equal to that of acetic acid to alcohol, unless no hydrogen whatever is produced. Since in this case the hydrogen formed only in the decomposition to pyruvic acid is completely used up for the reduction of acetaldehyde to ethyl alcohol, the surplus alcohol, *i.e.* the number of g.mols. of ethyl alcohol *minus* the number of g.mols. of acetic acid, must be equal to the number of g.mols. of carbon dioxide formed by the decomposition of pyruvic acid, provided that the latter is completely decomposed. Since pyruvic acid was never encountered among the products of fermentation, this may be safely assumed. From the sequel it will appear that under certain conditions this is indeed the case.

As regards the formation of succinic acid, it is assumed that this compound is produced directly from glucose by decomposition of the sugar molecule into two shorter chains containing two and four carbon atoms respectively. The arguments for this method of representation must also be omitted here.

That this fermentation scheme is a possible one has been shown by the various investigators. At this stage it is not proposed to go any more deeply into the matter than to indicate the two primary requirements of the scheme.

1. The sum of the carbon in the various decomposition products calculated as percentages on the carbon originally present in the fermented glucose must add up very nearly to 100.

2. Primarily 1 g.mol. of glucose affords 2 g.mol. of methylglyoxal hydrate. If the "formic acid scheme" is followed these 2 g.mol. of methylglyoxal hydrate are converted into 2 g.mol. of formic acid and 2 g.mol. of acetaldehyde hydrate, from which (assuming that the formic acid is completely decomposed) finally, 2 g.mol. each of carbon dioxide, hydrogen, acetic acid and alcohol will be produced.

If the "pyruvic acid scheme" is followed, 2 g.mol. of methylglyoxal hydrate are converted into 2 g.mol. of pyruvic acid and 2 g.mol. of hydrogen,

the pyruvic acid producing subsequently 2 g.mol. of carbon dioxide; the hydrogen, as in alcoholic fermentation, is used up in the reduction of acetaldehyde, formed as an intermediate, to ethyl alcohol.

As a matter of fact, however, the whole of the formic acid is never converted into hydrogen and carbon dioxide and some can be recovered after the fermentation. At the same time, part of the methylglyoxal hydrate is stabilised by conversion into lactic acid. The line of thought sketched very briefly above is sufficient to allow of the probability of the "reaction scheme" being tested against the experimentally observed facts by calculating the quantities of the products obtained at the conclusion of the experiment to corresponding amounts of hydrogen, carbon dioxide and acetaldehyde. If the scheme is "correct", then these quantities must be such that 100 g.mol. of hydrogen, carbon dioxide and acetaldehyde must correspond with 50 g.mol. of fermented glucose. In this calculation:

1 g.mol. of unchanged formic acid corresponds to 1 g.mol. of hydrogen *plus* 1 g.mol. of carbon dioxide;

1 g.mol. of ethyl alcohol will equal 1 g.mol. of acetaldehyde *plus* 1 g.mol. of hydrogen;

1 g.mol. of acetic acid will equal 1 g.mol. of acetaldehyde *minus* 1 g.mol. of hydrogen;

1 g.mol. of lactic acid will represent 1 g.mol. of acetaldehyde *plus* 1 g.mol. of hydrogen *plus* 1 g.mol. of carbon dioxide.

As regards the succinic acid, which occurs in the fermentation, this is considered to result from the direct degradation of glucose without previous phosphorylation. The glucose consumed in this manner will be prevented from taking part in any subsequent fermentation. To test the correctness of this assumption it is necessary for each g. of succinic acid formed to subtract $\frac{180}{118} \times 1$ g. from the amount of glucose decomposed during fermentation and to consider the remainder as "fermented glucose" in the above calculations. At the same time, 1 g.mol. of acetaldehyde for each g.mol. of succinic acid formed must be subtracted from the acetaldehyde balance, since acetaldehyde is formed along with succinic acid and is found in the final products of the fermentation either as alcohol or acetic acid, both of which appear in the acetaldehyde balance.

It needs hardly to be said that all these considerations only lead to a greater or smaller degree of probability of a given reaction-scheme. It is obvious that they do not form an exact proof of it.

For comparison, a few fermentations of glucose were carried out with growing *B. coli* cultures as well as with suspensions of "resting" *B. coli*. These fermentations took place under strictly anaerobic conditions in a caseinogen-peptone solution diluted with twice its volume of 0·5% sodium chloride solution. The original, undiluted caseinogen-peptone (again prepared according to Stickland's recipe [see Cole and Onslow, 1931]) presented considerable difficulty in the analyses of the fermented substrates. Since these experiments were carried out exactly as described in detail in earlier work, a reference to the relevant publication will suffice [Tasman and Pot, 1935]; here it need only be mentioned that 2% glucose was always used and 2% of previously sterilised chalk was added to fix the acids formed during fermentation.

Fermentations of glucose with *B. coli* suspensions were carried out as follows. 4·00 g. of glucose were weighed out and dissolved in 400 ml. of a phosphate buffer solution, p_H 6·2, in a pyrex flask with an external mark at 750 ml. The flask was closed by a doubly bored rubber stopper carrying a dropping funnel with a long stem reaching into the liquid and a rectangularly

bent exit tube also fitted with a tap. The dropping funnel and the exit tube were made germ tight by means of cotton-wool plugs. The contents of the flask were sterilised in the usual way for 12 min. at 115°, the dropping funnel being closed and the exit tube open. Directly after sterilisation, the second tap was closed and the flask connected, while still hot, to a cylinder of nitrogen. The nitrogen was freed completely from oxygen by passage through alkaline pyrogallol solution. Thus during the cooling, the flask filled itself with nitrogen. It was then placed in a thermostat at 40° and the exit tube connected with a train of three calcium chloride tubes followed by three tared soda-lime tubes. The gases passing from the soda-lime tubes were collected in a measuring cylinder over paraffin oil.

The bacterial cultures were centrifuged, those from liquid media directly, and those from solid media after previously shaking with salt solution, washed twice with saline and suspended in 200 ml. of phosphate buffer solution previously boiled to free it completely from carbon dioxide. This suspension was then introduced into the fermentation flask *via* the dropping funnel and the bulb of the latter washed out with 100 ml. of boiled phosphate buffer solution. When temperature had reached equilibrium, the paraffin was sucked into the measuring cylinder and the contents of the flask were periodically shaken. Fermentation of the glucose set in rapidly and was usually complete in about 5 hours, during which time usually not more than about 1·8 g. of glucose were fermented. The cause of this appears to lie in the fact that the p_H of the buffer solution falls to about 4·4–4·9 owing to the formation of the various acids (formic, acetic, lactic, succinic) during fermentation. For all practical purposes, glucose is no longer fermented at these low hydrogen ion concentrations. This fact is in complete agreement with the phenomena observed by Stephenson and Stickland. Since, however, the idea was to obtain glucose fermentation by "resting" *B. coli* under as nearly as possible the same conditions as in the experiments of Stephenson and Stickland, the addition of chalk was dispensed with, although without doubt this would have ensured the fermentation of greater amounts of glucose. Also since fermentation took place at different rates in the different experiments, analyses of the substrate were always carried out about 18 hours after the commencement of fermentation.

As regards the methods of analysis employed for the various glucose fermentations, reference may be made to an earlier paper on this subject [Tasman and Pot, 1934]; attention will be drawn here to a few particulars only.

The mixtures obtained by fermenting with suspensions were first of all freed from bacteria before the analysis by centrifuging for an hour.

In peptone fermentations the total *volatile acids* (acetic and formic acids) were determined in the way already described, but in fermentations with suspensions 200 ml. of clear, centrifuged liquid were submitted to a fractional steam-distillation, in which six 250 ml. fractions were collected and titrated separately, the titration of the last fraction giving the blank titration by which each previous titration of 250 ml. had to be reduced. The whole six fractions were evaporated down to about 50 ml. after the titrations and the formic acid was determined in the usual way by the calomel method.

The *alcohol determination* in fermented caseinogen-peptone substrates was carried out in liquid which had been previously freed from peptones by precipitation with phosphotungstic acid, since determinations made in untreated substrates gave too high results.

Since phosphoric acid is noticeably soluble in ether, the residue from the steam distillation was precipitated with magnesia mixture to free it from phosphates

prior to being evaporated down for the determination of lactic and succinic acids.

Lactic acid was determined by the method of Friedemann and Kendall, details of which have been published previously, in peptone fermentations, but with suspensions the oxalic acid method of Ulzer and Seidel was used [Tasman, 1932].

It is of course obvious that the same limits of accuracy cannot possibly be reached in the analytical results on fermentations with suspensions as are obtained in the case of fermentations with growing bacteria. In the first case, only about 1·5 g. of glucose are fermented against 20–35 g. in the second group of fermentations. Thus the fact, that in some cases the results are extremely good and the hydrogen/carbon dioxide and acetic acid/alcohol ratios, for example, are in remarkable agreement, will certainly be due more to accident than to actual experimental accuracy. This holds especially for determinations of acetic acid, formic acid and carbon dioxide, which certainly do not reach a high degree of accuracy in suspension fermentations.

Most of the fermentation experiments were carried out in duplicate. In order to save space, however, only one example of each type of glucose fermentation will be given.

I. *Fermentation of glucose by growing* B. coli *in caseinogen-peptone.*

These were carried out with strains 1452 and "Stickland", which were always kept on caseinogen-peptone-agar. The results of these experiments are collected together in Tables I and II.

A consideration of these results shows that both fermentations agree completely with those described by Scheffer for *B. coli* and by Tasman and Pot for strains of *B. paratyphosus*. The fermentation takes place chiefly *via* the "formic acid" route, while, more particularly in the case of the fermentation of glucose by the "Stickland" strain, the "pyruvic acid scheme" is followed to some extent. Thus it appears that the hydrogen comes in all probability chiefly from formic acid formed as an intermediate, along with a small amount from the decomposition of methylglyoxal hydrate into pyruvic acid and hydrogen.

Table I.

B. coli 1452. Growing in dilute caseinogen-peptone.

Products	g.	% of carbon in the products, calculated on the carbon present in the glucose fermented	g.mol. per 50 g.mol. fermented glucose. 5·54 g. of glucose and 8·9 g.mol. of acetaldehyde subtracted for each 3·63 g. of succinic acid		
			Hydrogen	Carbon dioxide	Acetaldehyde
Added glucose	36·60				
Glucose recovered	0·02				
Fermented glucose	36·58	100	Hydrogen	Carbon dioxide	Acetaldehyde
Hydrogen	0·220	—	31·9	—	—
Carbon dioxide	5·43	10·1	—	36·2	—
Acetic acid	4·88	13·3	23·6	—	23·6
Formic acid	0·232	0·4	1·5	1·5	—
Ethyl alcohol	4·25	15·2	26·8	—	26·8
Lactic acid	15·6	42·7	50·2	50·2	50·2
Succinic acid	3·63	10·1	—	—	− 8·9
	Total	91·8	86·8	87·9	91·7

Hydrogen/carbon dioxide = 0·89. Acetic acid/ethyl alcohol = 0·88.

Table II.

B. coli "Stickland". Growing in dilute caseinogen-peptone.

Products	g.	% of carbon in the products, calculated on the carbon present in the glucose fermented	g.mol. per 50 g.mol. fermented glucose. 1·36 g. of glucose and 3·1 g.mol. of acetaldehyde subtracted for each 0·89 g. of succinic acid		
			Hydrogen	Carbon-dioxide	Acet-aldehyde
Added glucose	34·60				
Glucose recovered	11·62				
Fermented glucose	22·98	100			
Hydrogen	0·125	—	26·2	—	—
Carbon dioxide	3·74	11·9	—	35·4	—
Acetic acid	2·98	13·2	−20·8	—	20·8
Formic acid	0·769	2·2	7·0	7·0	—
Ethyl alcohol	3·62	20·5	32·5	—	32·5
Lactic acid	11·0	47·9	50·9	50·9	50·9
Succinic acid	0·89	3·7	—	—	− 3·1
	Total	99·4	95·6	92·3	101·1

Hydrogen/carbon dioxide = 0·74. Acetic acid/ethyl alcohol = 0·65.

For comparison with these glucose fermentations an experiment was carried out in 1 % Witte peptone solution. In this case strain 3812 was used, which had never been cultivated on caseinogen-peptone (Table III).

Table III.

B. coli 3812. Growing in 1 % Witte-peptone.

Products	g.	% of carbon in the products, calculated on the carbon present in the glucose fermented	g.mol. per 50 g.mol. fermented glucose. 3·02 g. of glucose and 6·9 g.mol. of acetaldehyde subtracted for each 1·98 g. of succinic acid		
			Hydrogen	Carbon-dioxide	Acet-aldehyde
Added glucose	34·89				
Glucose recovered	9·84				
Fermented glucose	22·05	100			
Hydrogen	0·338	—	69·0	—	—
Carbon dioxide	6·47	17·6	—	60·1	—
Acetic acid	7·12	28·5	−48·5	—	48·5
Formic acid	1·25	3·3	11·1	11·1	—
Ethyl alcohol	4·78	24·9	42·5	—	42·5
Lactic acid	3·28	13·1	14·9	14·9	14·9
Succinic acid	1·98	8·4	—	—	− 6·9
	Total	95·8	89·0	86·1	99·0

Hydrogen/carbon dioxide = 1·15. Acetic acid/ethyl alcohol = 1·13.

From the above it appears that under these conditions the "formic acid scheme" is followed exclusively, whilst at the same time the relatively large amount of hydrogen and carbon dioxide which is formed is remarkable. Thus here the hydrogen is derived probably from the intermediate formic acid.

It is self-evident in the light of what has previously been stated, that in the three fermentation experiments mentioned above the formation of succinic acid can be explained by a fission of the glucose molecule into groups containing two and four atoms of carbon.

II. *Fermentation of glucose by "resting" B. coli in a phosphate buffer; the suspension prepared by partial anaerobic culture in liquid caseinogen-peptone.*

Subcultures of strains 1452 and "Stickland" were made in caseinogen-peptone. At the end of 24 hours 16 bottles of 1000 ml. capacity and each containing 500 ml. of caseinogen-peptone were inoculated with the subcultures, and after about 20 hours a suspension of this culture was made in the way mentioned previously and used for the glucose fermentation experiments. The results of these experiments are collected together in Tables IV and V.

Table IV.

B. coli 1452. Cultivation: partially anaerobic in liquid caseinogen-peptone. "Resting" *B. coli* suspension in phosphate buffer p_H 6·2.

Products	g.	% of carbon in the products, calculated on the carbon present in the glucose fermented	g.mol. per 50 g.mol. fermented glucose. 0·49 g. of glucose and 22 g.mol. of acetaldehyde subtracted for each 0·32 g. of succinic acid		
		100	Hydrogen	Carbon-dioxide	Acet-aldehyde
Added glucose	4·00				
Glucose recovered	2·38				
Fermented glucose	1·62				
Hydrogen	0·010	—	41	—	—
Carbon dioxide	0·30	13	—	54	—
Acetic acid	0·17	10	−22	—	22
Formic acid	0·014	0·6	2·4	2·4	—
Ethyl alcohol	0·18	14	31	—	31
Lactic acid	0·59	37	52	52	52
Succinic acid	0·32	20	—	—	−22
	Total	94·6	104·4	109·4	83

Hydrogen/carbon dioxide = 0·75. Acetic acid/ethyl alcohol = 0·72.

Table V.

B. coli "Stickland". Cultivation: partially anaerobic in liquid caseinogen-peptone. "Resting" *B. coli* suspension in phosphate buffer p_H 6·2.

Products	g.	% carbon in the products, calculated on the carbon present in the glucose fermented	g.mol. per 50 g.mol. fermented glucose. 0·23 g. of glucose and 9·8 g.mol. of acetaldehyde subtracted for each 0·15 g. of succinic acid		
		100	Hydrogen	Carbon-dioxide	Acet-aldehyde
Added glucose	4·00				
Glucose recovered	2·62				
Fermented glucose	1·38				
Hydrogen	0·0039	—	16	—	—
Carbon dioxide	0·29	14	—	51	—
Acetic acid	0·056	4	−7·2	—	7·2
Formic acid	0·0053	0·2	0·9	0·9	—
Ethyl alcohol	0·15	14	25	—	25
Lactic acid	0·74	51	64	64	64
Succinic acid	0·15	10	—	—	− 9·8
	Total	93·2	98·1	115·9	86·4

Hydrogen/carbon dioxide = 0·29. Acetic acid/ethyl alcohol = 0·29.

The following may be concluded from these results. Strain 1452 in suspension, *i.e.* as "resting" *B. coli*, ferments glucose in practically the same way as

when growing bacteria are used and chalk is added to the medium to fix the acids which are produced (see Table I). At the most, rather more is decomposed according to the "pyruvic acid scheme". Yet even under these conditions it may be assumed that the greater part of the evolved hydrogen originates from formic acid produced during the fermentation of the glucose.

On the other hand, the "Stickland" strain behaves otherwise. Here, apparently, the "pyruvic acid scheme" predominates, since the ratios hydrogen/carbon dioxide and acetic acid/ethyl alcohol, which are remarkably closely equal to one another, deviate very considerably from unity. However, since very little formic acid remains in the substrate (compare glucose fermentations with aerobically grown cultures discussed below, Tables VII and VIII), it may be assumed that in these glucose fermentations the hydrogen liberated as gas may still have its origin in the intermediate product, formic acid.

III. *Fermentations of glucose by "resting"* B. coli *in phosphate buffers. Suspensions prepared by partially anaerobic culture in caseinogen-peptone* plus *1% of glucose.*

In connection with the possibility that *B. coli* might be able to ferment glucose in another way if it were "adapted" to this purpose beforehand, strain 1452 was subcultured daily for a fortnight on slopes of caseinogen-peptone-agar to which 0·5% of glucose had been added. A glucose fermentation was carried out with a suspension of this culture prepared in the usual way by subculture in liquid caseinogen-peptone containing 1% of glucose, and the results are found in Table VI.

Table VI.

B. coli 1452. Cultivation: before the experiment the strain was subcultured daily for 14 days on caseinogen-peptone-agar containing 0·5% glucose. A suspension was then prepared by partial anaerobic cultivation in liquid caseinogen-peptone containing 1% of glucose. "Resting" *B. coli* suspension in phosphate buffer p_H 6·2.

Products	g.	% of carbon in the products, calculated on the carbon present in the glucose fermented	g.mol. per 50 g.mol. fermented glucose		
			Hydrogen	Carbon-dioxide	Acet-aldehyde
Added glucose	4·00				
Glucose recovered	2·65				
Fermented glucose	1·35	100	Hydrogen	Carbon-dioxide	Acet-aldehyde
Hydrogen	0·0086	—	29	—	—
Carbon dioxide	0·022	11	—	34	—
Acetic acid	0·082	6·1	- 9·3	—	9·3
Formic acid	0·0025	0·1	0·4	0·4	—
Ethyl alcohol	0·093	9·0	13	—	13
Lactic acid	1·0	75	74	74	74
Succinic acid	—	—	—	—	—
Total		101·3	107·1	108·4	96·3

Hydrogen/carbon dioxide = 0·84. Acetic acid/ethyl alcohol = 0·70. p_H of fermented liquid = 4·41.

When these figures are compared with those in Table IV, in which the corresponding glucose fermentation was carried out with the same strain which had not however been "adapted" to glucose, then the only difference which comes to light is the absence of succinic acid from the products of fermentation, while approximately twice the usual amount of lactic acid is formed. In agreement with this, the other products of fermentation were found in relatively smaller quantities.

In both cases, however, the hydrogen/carbon dioxide and the acetic acid/alcohol ratios are practically equal, so that here again it may be assumed with great probability that the gaseous hydrogen is liberated *via* formic acid.

IV. *Fermentation of glucose by "resting" B. coli in a phosphate buffer. Suspension prepared by completely aerobic culture on caseinogen-peptone-agar.*

Roux flasks were used for the preparation of the necessary bacterial material, each flask containing a layer of solidified caseinogen-peptone-agar (4% agar) which was inoculated with a culture in liquid caseinogen-peptone. The results of a glucose fermentation carried out with this suspension are given in Table VII.

Table VII.

B. coli 1452. Cultivation: completely aerobic culture on caseinogen-peptone-agar. "Resting" *B. coli* suspension in phosphate buffer p_H 6·2.

Products	g.	% of carbon in the products, calculated on the carbon present in the glucose fermented	g.mol. per 50 g.mol. fermented glucose. 0·088 g. of glucose and 5·6 g.mol. of acetaldehyde subtracted for each 0·058 g. of succinic acid		
			Hydrogen	Carbon-dioxide	Acet-aldehyde
Added glucose	4·00				
Glucose recovered	3·12				
Fermented glucose	0·88	100			
Hydrogen	—	—	—	—	—
Carbon dioxide	0·047	3·6	—	12	—
Acetic acid	0·067	7·8	−13	—	13
Formic acid	0·19	14	47	47	—
Ethyl alcohol	0·10	18	25	—	25
Lactic acid	0·49	55	61	61	61
Succinic acid	0·058	6·2	—	—	− 5·6
	Total	104·6	120	120	93·4

Acetic acid/ethyl alcohol = 0·49.

Surplus ethyl alcohol $= \frac{0{\cdot}10}{46} - \frac{0{\cdot}067}{60} = 0{\cdot}00217 - 0{\cdot}00110 = 0{\cdot}00107$ g.mol.

Carbon dioxide produced $= \frac{0{\cdot}047}{44} = 0{\cdot}00107$ g.mol.

V.P.—reaction negative.

p_H of the fermented liquid = 4·75.

It is at once obvious that hydrogen formation is completely absent, which is contrary to the qualitative experiments described in the first communication (see the Table, Exps. 9 and 13, strain 1452). In how far the undoubtedly different conditions under which these experiments were carried out determined the results must be left to conjecture. It may be mentioned here, however, that in the series of experiments described previously the formation of hydrogen from glucose under these conditions was in no way regular (see Exp. 12 in which the bacteria were cultivated on caseinogen-peptone-agar (puriss. Grübler)).

Against the absence of hydrogen from the fermentation products, a relatively large amount of formic acid and a small amount of carbon dioxide must be noted. The question naturally arises as to where this carbon dioxide originates. From the fact that this *B. coli* strain, 1452, under all conditions follows the "formic acid scheme" to the greater extent, yet at the same time always follows the "pyruvic acid scheme" to a much less extent, it is obvious that the reason for

the formation of carbon dioxide in the case in question is to be sought in the decomposition of glucose in the latter way. If this supposition is correct, the whole of the hydrogen resulting from the decomposition of methylglyoxal hydrate must be completely used up in the reduction of acetaldehyde to alcohol. Consequently, the quantities of carbon dioxide and excess alcohol (*i.e.* alcohol *minus* acetic acid) formed must be equivalent when expressed in g.mol. This appears indeed to be the case. That these quantities are so nearly equal in this case must of course be ascribed to an accidentally very favourable analytical result.

The fact that the quantities of hydrogen and carbon dioxide occurring in the hydrogen-carbon dioxide-acetaldehyde balance deviate from 100 is caused in all probability by the formic acid determination giving too high results. The amount of glucose which undergoes fermentation is so small that the fermentation balance, based as it is on these analyses, cannot be very accurate and further explanation is unnecessary.

That so little glucose is fermented under these conditions readily finds an explanation in the fact that the formic acid formed is not decomposed further so that the hydrogen ion concentration falls much more rapidly to the fatal low limit than would otherwise be the case, which must again result in still less glucose being fermented.

V. *Fermentation of glucose by "resting" B. coli in a phosphate buffer. Suspension prepared by complete aerobic culture on caseinogen-peptone plus 0·5% of sodium formate.*

Finally, it was investigated how far a previous "acclimatisation" to formic acid under aerobic conditions would enable the bacterial suspension to decompose

Table VIII.

B. coli 1452. Cultivation: before the experiment the strain was subcultured daily for 14 days on caseinogen-peptone-agar containing 0·5% of sodium formate. A suspension was then made by complete aerobic cultivation on caseinogen-peptone *plus* 0·5% sodium formate. "Resting" *B. coli* suspension in phosphate buffer p_H 6·2.

Products	g.	% of carbon in the products, calculated on the carbon present in the glucose fermented	g.mol. per 50 g.mol. fermented glucose. 0·20 g. of glucose and 16 g.mol. of acetaldehyde subtracted for each 0·13 g. of succinic acid		
			Hydrogen	Carbon-dioxide	Acet-aldehyde
Added glucose	4·00				
Glucose recovered	3·18				
Fermented glucose	0·82	100	Hydrogen	Carbon-dioxide	Acet-aldehyde
Hydrogen	—	—	—	—	—
Carbon dioxide	0·027	2·2	—	8·5	—
Acetic acid	0·080	9·8	−20	—	20
Formic acid	0·19	15	59	59	—
Ethyl alcohol	0·080	13	26	—	26
Lactic acid	0·41	50	66	66	66
Succinic acid	0·13	16	—	—	−16
	Total	106	131	131·5	96

Acetic acid/ethyl alcohol = 0·75.

Surplus ethyl alcohol $= \frac{0·080}{46} - \frac{0·080}{60} = 0·00174 - 0·00133 = 0·0041$ g.mol.

Carbon dioxide produced $= \frac{0·027}{44} = 0·0061$ g.mol.

p_H of the fermented liquid = 4·89.

glucose with the formation of hydrogen. For this purpose, strain 1452 was subcultured daily for a fortnight on caseinogen-peptone-agar *plus* 0·5 % of sodium formate and inoculated into Roux flasks containing caseinogen-peptone-agar *plus* 0·5 % of sodium formate *via* a passing subculture. The result of a fermentation carried out with this suspension is given in Table VIII.

From this result it appears that the "acclimatisation" did not have the desired effect. The experiment agrees essentially with those carried out previously. In this case also decomposition took place mainly *via* the "formic acid scheme" and only to a small extent *via* the "pyruvic acid scheme". Here again the hydrogen is in all probability used up completely in the reduction of acetaldehyde to ethyl alcohol, which follows from the fact that the values for the surplus alcohol and carbon dioxide formed are about equal when expressed in g.mol. That the agreement in this case is not so close as in the previous experiment needs no further explanation. The small amount of glucose undergoing fermentation is explained as mentioned earlier.

Discussion.

Reviewing the whole of these experiments we find a quantitative confirmation of the experiments described in the first communication which were only of a qualitative nature, and little need be added to the conclusions formulated therein.

In conclusion, as regards the decomposition of glucose by *B. coli* under anaerobic conditions, it may be assumed that in the majority of cases this decomposition takes place *via* the "formic acid scheme" and the hydrogen liberated in the gaseous state is formed mainly by the decomposition of formic acid produced as an intermediate. Only in a few cases does the decomposition of glucose *via* the "pyruvic acid scheme" occur to a preponderating extent. The same remarks hold for fermentations of glucose both with living reproductive *B. coli* and in those cases where the glucose was fermented in a phosphate buffer at p_H 6·2 with a suspension of "resting" *B. coli*. All the phenomena observed are in agreement with the reaction scheme put forward by Kluyver, Scheffer *et al.*, for the fermentation of glucose by members of the coli-typhosus-dysentericus group.

Summary.

1. In connection with experiments on the formation of hydrogen and carbon dioxide in the fermentation of glucose by "resting" *B. coli*, which have been described in an earlier paper, experiments are described in the present communication in which glucose has been fermented under anaerobic conditions both by living reproductive *B. coli* and with suspensions of "resting" *B. coli*.

2. In the majority of cases, the glucose is decomposed mainly in conformity with the so-called "formic acid scheme", showing that in all probability the gaseous hydrogen produced in these cases is formed by the decomposition of formic acid, occurring as an intermediate, into equivalent amounts of hydrogen and carbon dioxide.

3. Only in a few cases is the glucose decomposed mainly in accordance with the so-called "pyruvic acid scheme". This is expressed in the fact that although the ratios hydrogen/carbon dioxide and acetic acid/alcohol are equal, they deviate considerably from unity.

4. All the observed facts appear to be in agreement with the reaction scheme for the fermentation of glucose put forward by Kluyver, Scheffer and their co-workers.

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