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

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DURING recent years Stickland [1929] and Stephenson and Stickland [1931; 1932; 1933] have published a series of investigations on the decomposition of glucose and formic acid by the so-called "resting" *B. coli*. They concentrated their attention chiefly on the question of the evolution of hydrogen in this reaction and came to the conclusion that their bacterial preparations (*B. coli* grown on caseinogen or broth, centrifuged, washed and suspended in buffer solutions) must have contained three different enzymes.

(1) *Dehydrogenase*, an enzyme, which activates the hydrogen atoms in the substrate molecule, provided a suitable hydrogen acceptor is present simultaneously. For example, formic acid dehydrogenase catalyses the reaction $H.COOH + R \rightleftharpoons RH_2 + CO_2$. In this case methylene blue can serve as a hydrogen acceptor.

(2) *Hydrogenase*, a ferment, which under the same conditions activates molecular hydrogen present in a substrate and conveys it to a suitable hydrogen acceptor. This reaction can be represented by the equations $H_2 \rightleftharpoons 2H$, and $2H + R \rightleftharpoons RH_2$. Here again methylene blue or even molecular oxygen may act as acceptor.

(3) *Hydrogenlyase*, which generates molecular hydrogen from formic acid or glucose in the absence of an acceptor. This reaction is represented as follows for the decomposition of formic acid: $H.COOH \rightleftharpoons H_2 + CO_2$.

However, the authors are not quite consistent in their nomenclature. In one case [1931, p. 213] the decomposition of formic acid into molecular hydrogen and carbon dioxide was ascribed to the combined action of the two enzymes formic dehydrogenase and hydrogenase, while later [1932, p. 715] the same action was supposed to result from formic hydrogenlyase. However, this probably only points to a justifiable change of opinion during the carrying out of the research and in any case it is not of great importance.

The cardinal point in the publications of these authors from our point of view is the fact that they make an important distinction between the formation of molecular hydrogen from glucose and from formic acid, the responsibility for which they ascribe to the enzymes glucose hydrogenlyase and formic hydrogenlyase, respectively. Thus the formation of hydrogen from glucose does not occur *via* formic acid, which is always produced in the fermentation with *B. coli*, but represents an essentially different process.

Like Dienert [1900], Karström [1930] differentiates between constitutive and adaptive enzymes. The former are present all the time in the bacterial cell, independently of the mode of cultivation; the adaptive enzymes, however, are formed only in response to a special stimulus during growth exerted in one medium or another by the substance, which will be decomposed (by "resting" microbes) in subsequent experiments.

Yudkin [1932] has extended this idea somewhat by speaking of constitutive enzymes and those obtained by pure "adaptation" or by "training". The difference between the last two categories is that an enzyme obtained by "training" (*i.e.* cultivation for a more or less extended time in a suitable medium) does not lose or at least only slowly loses, its characteristics when successive generations of the organism are bred in a medium from which the stimulating substance is absent, whilst an adaptive enzyme very soon disappears when the organism is cultivated in a medium without a special enzyme regulator. According to these ideas glucose hydrogenlyase is a constitutive and formic hydrogenlyase an adaptive enzyme.

This essential differentiation between the enzymes, which liberate hydrogen from glucose and formic acid, respectively, is justified on the grounds of the following facts according to Stephenson and Stickland.

(1) When *B. coli* is grown under certain conditions (more or less anaerobic) on caseinogen broth without glucose or formate, then centrifuged, washed and suspended in a suitable phosphate buffer, this suspension produces gas containing hydrogen from glucose but does not attack formate. Only when formate or glucose is present previously (the latter forms formate on decomposition) has a bacterial suspension prepared as stated above the property of decomposing both glucose and formate with the production of gas.

(2) The optimum p_H for the two enzymes is different, *viz.* 6·2 for glucose hydrogenlyase and 7·0 for the formation of hydrogen from formic acid.

(3) The affinity of the two enzymes towards substrates is unequal, being much greater for glucose hydrogenlyase than for the enzyme which breaks up formic acid.

(4) The formation of hydrogen from glucose occurs instantly without any lag period and takes place linearly, which according to Stephenson and Stickland would not be expected if the formation of hydrogen from glucose took place *via* formic acid.

They state their final conclusion as follows:

"The present state of our knowledge on this point is therefore that the hydrogen from glucose does not come through formic acid and that it is liberated by an enzyme, which is not formic hydrogenlyase; whether it is liberated direct from the glucose molecule or from some other intermediate compound we do not know, though the absolute linearity of the reaction indicates that the former may be true."

A summary of these investigations containing the above conclusion has been given by Miss Stephenson [1933].

Since this view of the decomposition of glucose and the method by which hydrogen is formed is not only contrary to the generally accepted theory of this phenomenon but is also at variance with our own experiments on the fermentation of glucose by both gas-forming and non-gas-forming paratyphoid bacilli, it seemed to us that the question was worth reinvestigation.

In the first place we have investigated how far it is possible to prepare suspensions of *B. coli* which will generate hydrogen from glucose but not from formic acid. The same technique was followed in the preparation of these suspensions as was employed by Stephenson and Stickland.

At first we used Witte peptone as the source of nitrogen in our culture media but later we also employed caseinogen-peptone. The preparation of the latter product was carried out according to directions supplied personally by Stickland. This recipe agrees practically completely with that of Cole and Onslow [1931]. It consists in a digestion of caseinogen dissolved as sodium salt by a very

powerful trypsin preparation. Unless otherwise stated, caseinogen broth was always prepared from cheap commercial caseinogen.

Our first strain of *B. coli* (261) was isolated by allowing a drop of faeces suspension to grow on peptone-agar plates (1 % Witte peptone, 0·5 % sodium chloride and 2 % agar in tap-water) and examining the properties of several transplanted colonies. The second strain (1452) was isolated in exactly the same way on caseinogen-peptone. The *B. coli* strain marked "Stickland" was received from Stickland to whom we again tender our thanks. This strain was originally taken from the National Collection of Type-cultures in London and in all probability, therefore, had been subcultured for several years in artificial culture media. The last two strains (3812 and 3813) were isolated like the first on Witte peptone-agar. The properties of all strains were periodically examined in the usual way.

Since Stephenson and Stickland stated that they allowed their *B. coli* cultures to grow in liquid caseinogen broth in Roux flasks, *i.e.* under practically complete aerobic conditions, while Yudkin on the other hand states that under strictly aerobic conditions glucose hydrogenlyase is formed only to a very slight extent and formic hydrogenlyase is not formed at all, it appeared to us not improbable that the production of the two enzymes was more or less dependent on the aerobic or anaerobic condition of the medium during growth.

Our experiments were carried out in the following way:

(1) *Method of cultivation.*

(*a*) Aerobic on Witte peptone-agar.

(*b*) Aerobic on Witte peptone-agar to which 1 % of glucose was added.

(*c*) Aerobic on Witte peptone-agar to which 1 % of calcium formate was added.

(*d*) For the greater part anaerobic in Witte peptone solution (1 % peptone, 0·5 % sodium chloride in tap water).

(*e*) Like (*d*) in 3 % Witte peptone broth.

(*f*) Like (*d*) in liquid caseinogen-peptone.

(*g*) Aerobic on caseinogen-peptone-agar.

(*h*) Aerobic on caseinogen-peptone-agar starting from very pure "Casein-puriss" Grübler.

(*j*) Like (*d*) in liquid "Casein-puriss" Grübler-peptone.

(2) *Preparation of the suspensions.*

After 20–24 hours' incubation the liquid cultures were centrifuged and the solid cultures were suspended in physiological salt solution and then centrifuged. The precipitates so obtained were washed twice with salt solution and finally suspended in a small volume of this liquid.

Since all our *B. coli* strains grew much better on or in caseinogen-peptone than on or in Witte peptone, the suspensions obtained in the former culture medium were usually thicker than those obtained with Witte peptone. However, since the results given below are only of a qualitative character, we did not go to the trouble of diluting all our suspensions to the same density.

(3) *Fermentation experiments.*

Stephenson, Stickland and Yudkin used the accurate manometric method, particulars of which are given by Dixon [1934], in their experiments, which were of a quantitative nature. Since we had no such apparatus available, we had to

be content with observing the formation or the non-formation of gas from glucose or formate in the well-known Einhorn fermentation tubes. For this purpose, 15 ml. of the suspension in question were mixed with 15 ml. of 1% sodium formate solution in a phosphate buffer at p_H 7·0 and 15 ml. of the suspension were mixed with 15 ml. of 1% glucose solution in a phosphate buffer at p_H 6·2. The formate and glucose solutions were previously sterilised for 12 mins. at 115°, which causes no loss or decomposition of the sugar. These mixtures were then filled into 10 ml. Einhorn tubes, which were placed in an incubator at 40°. The final result of the fermentation, *i.e.* the formation or non-formation of gas was noted after 24 hours. It was established that when gas was formed, it always consisted of a mixture of hydrogen and carbon dioxide. On account of the purely qualitative nature of these experiments no attempt was made to determine the exact amounts of gas produced but a rough idea of these quantities is given in the following ways:

− no formation of gas. tr, formation of a trace of gas. + definite formation of gas. ++ to ++++, the formation of considerable to excessive amounts of gas. The results of duplicate experiments are given in Table I.

Table I.

			Strain 261				Strain 1452			
			Glucose		Formate		Glucose		Formate	
No.	Date	Medium	1	2	1	2	1	2	1	2
1	7. ii. 34	1% Witte peptone-agar	+	+	−	−	.	.	.	.
2	10. ii. 34	1% Witte peptone-agar, 1% glucose agar	+	+	−	−	.	.	.	.
3	10. ii. 34	1% Witte peptone, 1% calcium formate-agar	+	+	−	−	.	.	.	.
4	21. ii. 34	Do.[1]	+	+	+	+	.	.	.	.
5	3. vi. 34	Liquid caseinogen-peptone	.	.	.	.	++	++	+++	+++
6	6. vi. 34	Do.	++	++	+++	+++	.	.	.	.
7	8. vi. 34	1% liquid Witte peptone	+	+	−	−	+	+	tr.	tr.
8	10. vi. 34	3% liquid Witte peptone	+	+	++	++	+	+	++	++
9	18. vi. 34	Caseinogen-peptone-agar	++	++	+++	+++	++	++	+++	+++
10	28. vi. 34	3% Witte peptone-agar[2]	−	−	++	++	tr.	−	++	++
11	7. vii. 34	1% Witte peptone-agar	−	tr.	+	++	+	−	+++	+++
12	17. vii. 34	Caseinogen-peptone (puriss. Grübler)-agar[3]	−	−	++	++	−	−	+++	+++
13	18. vii. 34	Liquid caseinogen-peptone (puriss. Grübler)	+	+	+	+	++	++	+++	+++
14	21. vii. 34	1% liquid Witte peptone	+	+	−	−	+	+	−	−
15	21. vii. 34	Liquid caseinogen-peptone	++	++	++++	++++	++	++	++++	++++
16	21. vii. 34	Caseinogen-peptone-agar	++	++	+++	+++	+	+	++	++
17	4. iv. 35	Witte peptone-agar	.	.	.	.	.	.	.	.
18	12. iv. 35	Do.[4]	.	.	.	.	.	.	.	.
19	25. iv. 35	Caseinogen-peptone-agar[5]	.	.	.	.	.	.	.	.

[1] This strain previously passed nine times through calcium formate.
[2] Strains 261 and 1452 had already been cultivated several times on caseinogen-peptone.
[3] Eight previous transfers on caseinogen-peptone puriss.

In this connection the following remarks should be made:

(1) All suspensions of all strains obtained by growing on caseinogen-peptone ferment formate with the production of gas.

(2) With one exception (No. 12), the same suspensions also produce gas from glucose. In Exp. 13, strain 261, the amount of gas formed with glucose was equal to that formed from formate; in all other experiments gas was produced more quickly and in larger quantities in the formate tubes than in those containing glucose. Since the English investigators always used caseinogen-peptone as the basis of their media, their results are in marked contrast to our own. Since we prepared our caseinogen-peptone exactly in accordance with Stickland's recipe

and also since we found no essential difference between caseinogen-peptone prepared from crude commercial caseinogen and that prepared from very pure "Casein puriss. Grübler," we are not able to explain this discrepancy. It should be stated that our caseinogen-peptone was always free from reducing sugars (lactose or glucose) and formate.

(3) Suspensions prepared with media made from Witte peptone behaved rather differently from those discussed above. The first three experiments (1, 2 and 3) with strain 261 gave the impression that in this case we were dealing with a strain which answered to the type used by Stephenson and Stickland. In these experiments gas was definitely produced from glucose but in very small quantities whilst in the case of formate gas was absent. However, when this strain was transferred to a caseinogen-peptone medium, gas was always formed from formate. If this strain was then transferred again to Witte peptone-agar, (Exps. 10 and 11) it no longer showed its original properties. When these experiments were repeated on strains 3812 and 3813, which were freshly isolated from Witte peptone, we succeeded in obtaining an equal or larger formation of gas from glucose than from formate, though in the latter case it was not absent

Table I (*cont.*).

Strain "Stickland"				Strain 3821				Strain 3813			
Glucose		Formate		Glucose		Formate		Glucose		Formate	
1	2	1	2	1	2	1	2	1	2	1	2
.	.	.	.	.	.	.	.	.	.	.	.
.	.	.	.	.	.	.	.	.	.	.	.
.	.	.	.	.	.	.	.	.	.	.	.
.	.	.	.	.	.	.	.	.	.	.	.
.	.	.	.	.	.	.	.	.	.	.	.
.	.	.	.	.	.	.	.	.	.	.	.
.	.	.	.	.	.	.	.	.	.	.	.
++	++	+++	+++	.	.	.	.	.	.	.	.
tr.	tr.	−	−	.	.	.	.	.	.	.	.
+	−	+++	+++	.	.	.	.	.	.	.	.
−	tr.	++	++	.	.	.	.	.	.	.	.
+	+	+	+	.	.	.	.	.	.	.	.
+	+	tr.	tr.	.	.	.	.	.	.	.	.
+	+	++++	++++	.	.	.	.	.	.	.	.
++	++	+++	+++	.	.	.	.	.	.	.	.
.	.	.	.	+++	+++	++	++	.	.	.	.
.	.	.	.	.	.	.	.	++	++	++	++
.	.	.	.	++	++	++++	++++	+	+	++++	++++

[4] After about 4 hours more gas was produced from glucose than from formate, but at the end of 24 hours equal amounts had been produced.

[5] Strain 3812 previously subcultured nine times and strain 3813 four times on caseinogen-peptone.

(Exps. 17 and 18). When the same strains were subsequently inoculated on caseinogen-peptone-agar (Exp. 19), the formation of gas from formate was again much larger than from glucose.

When grown on liquid Witte peptone (1 %) (Exps. 7 and 14) gas was produced from glucose but none or at most only a trace was formed from formic acid. However, Exp. 10 in which a 3 % Witte peptone solution was used, showed a distinct difference. Fermentations carried out with this suspension agreed entirely with those in which caseinogen-peptone was used as the culture medium.

Surveying the whole of the above experiments we came to the conclusion that by cultivating in or on caseinogen-peptone media we were not able to

obtain suspensions of *B. coli* free from "formic hydrogenlyase", as should have been the case according to Stephenson and Stickland, although in none of these experiments was formate or glucose present in the media used.

The results obtained with experiments with Witte peptone, although less uniform in character, definitely do not point in the opposite direction.

Stephenson and Stickland showed that the decomposition of formic acid took place much more rapidly if the cells were able to multiply rapidly, be it on account of the presence of peptone in the medium or at the expense of dead and autolysed bacteria. One might make the suggestion, therefore, that the formation of gas from formate in our experiments is due to the growth of the micro-organisms in question, but since evolution of gas with formate sets in immediately in all caseinogen-peptone experiments, very often before the Einhorn tubes could be placed in the incubator, this possibility may be disregarded.

In 1931 one of us [Pot, 1932; 1933] isolated, in the course of routine investigation in the bacteriological section of the late Central Laboratory, three strains of *B. paratyphosus*-B, which behaved serologically and biochemically as normal *paratyphosus* strains except in one particular, namely, that they did not possess the property of forming gas from glucose. We discovered a simple means of restoring to such strains of *paratyphosus* bacilli the power of liberating gas from glucose and formic acid by cultivating them for a fairly long period in a medium containing calcium formate [Pot and Tasman, 1932; 1933, 1, 2]. When gas was formed from calcium formate the power of liberating gas from glucose returned simultaneously.

During this investigation a detailed quantitative study was also made of the fermentation of glucose [Tasman and Pot, 1934]. Without going more deeply into the matter, suffice it to say that all strains encountered during the investigation ("non-gaseous" and similar types of *B. paratyphosus* made gas-forming by passage through calcium formate, as well as control strains of *B. typhosus* and *B. paratyphosus*) broke down glucose in essentially the same way but with this one difference that *typhosus* and "non-gaseous" *paratyphosus* strains were unable to decompose the formic acid formed in the fermentation any further, whilst normal and "non-gaseous" types of *B. paratyphosus*, which had been converted into gas-forming types, were able to bring about this decomposition. From these experiments it was most clearly apparent, that in the fermentation of glucose with growing and reproducing bacteria, the liberated hydrogen was completely, or practically completely, derived from the decomposition of formic acid produced as an intermediate (the so-called "formic acid scheme").

At the same time it was demonstrated that under certain conditions a very small proportion of the liberated hydrogen finds its origin in methylglyoxal hydrate, which very probably occurs as an intermediate product in the fermentation of glucose and which breaks down into pyruvic acid and hydrogen (the so-called "pyruvic acid scheme"). The results appear to be in complete agreement with the experiments and conclusions of Scheffer [1928], who investigated in detail the fermentation of glucose by various other representatives of the *coli-typhosus-dysentericus* group.

Thus if there are two enzymes, which are responsible for the formation of hydrogen from glucose, our "non-gaseous" strains should be deficient in both. If they acquire formic acid hydrogenlyase by passage through calcium formate, then at the same time they acquire glucose hydrogenlyase. Thus we must assume that the formate stimulus brings about a simultaneous regulation of both enzymes. This is also contrary to the ideas of Karström, who considers

glucose hydrogenlyase to be a constitutive and formic acid hydrogenlyase to be an adaptive enzyme.

Still another possibility is, that only formic acid hydrogenlyase is regulated by the formic acid stimulus and that now the gas from glucose, contrary to what is usually assumed to be the case in a normal fermentation, is actually formed from formic acid. We must then assume that only our *paratyphosus* strains, which have been converted into gas-forming types, produce hydrogen in the fermentation of glucose by decomposition of formic acid and that "normal" *paratyphosus* and *coli* strains must follow another fermentation scheme in spite of the fact that we were able to establish numerous fermentation balances for these decompositions of sugar by the various strains, which failed to show any essential difference whatever; an assumption which certainly possesses no great degree of probability.

Also Stephenson, Stickland and Yudkin state certain facts, which are not in agreement with their theoretical considerations. The former investigators state that a suspension grown in caseinogen broth with formate develops hydrogen from formate at p_H 7·0 with the same velocity as it is developed from glucose at p_H 6·2; a suspension grown on formate-free caseinogen-peptone, however, does not ferment formate and produces hydrogen from glucose but only at a rate about 15 % of that of the previous suspension. Besides they also found that the production of hydrogen from glucose was always accompanied by the formation of an equivalent amount of carbon dioxide. This points to the formation of this hydrogen from formic acid, which is formed as an intermediate, in which case equal amounts of hydrogen and carbon dioxide must be produced.

Yudkin noted that cultivation on media containing formate always increased the power of producing hydrogen from glucose, although not to the same degree as cultivation in a medium containing glucose. He can only form a picture of this reaction with difficulty because, according to his ideas, the decomposition of formic acid into hydrogen and carbon dioxide is not a reaction from which the living bacterial cell can derive energy. We have shown, however [Pot and Tasman, 1933, 2; 1934], that this fission of the molecule certainly puts energy at the disposal of the cell, which is also a plausible explanation of the fact that a culture of non-gas-producing *paratyphosus* bacteria always shows a large increase in the number of living organisms at the moment when it acquires the power of decomposing formic acid.

Thus, as a result of our experiments and theoretical considerations, we must first assume that the hydrogen produced in the decomposition of glucose by growing and "resting" *coli* bacteria, is, in the majority of cases, the result of the decomposition of formic acid produced as an intermediate. Simultaneously, in a few cases, a small quantity of hydrogen may originate from the decomposition of glucose according to the "pyruvic acid scheme", but the formation of hydrogen in this manner is mostly insignificant.

Further, we consider it undesirable to assume the presence of different enzymes. In this particular we take the standpoint of Kluyver [1931] who utters the strongest possible warning against assuming the presence of new, so-called "specific", enzymes for the various oxidation-reduction reactions or hydrogen activation reactions, which seem to make their appearance in the different decomposition reactions.

Finally, we wish to state that in a subsequent publication we hope to elucidate the fermentation of glucose by "resting" *B. coli* from a quantitative standpoint by setting up fermentation balances.

SUMMARY.

1. On repeating the experiments of Stephenson, Stickland and Yudkin, that is to say by growing bacilli on the same media as they used, we have not been able to confirm their results with regard to the decomposition of glucose and formic acid by "resting" *B. coli*.

2. On the basis of these experimental results and from theoretical considerations we come to the conclusion that the formation of hydrogen from glucose occurs, certainly in the majority of cases, *via* formic acid, which is produced as an intermediate compound, and only under certain conditions, in a small proportion of cases, is it produced by other means.

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