CXI. FERMENTATION OF SUGAR BY THE ROOT NODULE BACTERIA[1].

By ARTTURI ILMARI VIRTANEN, MANNE NORDLUND and ELIAS HOLLO.

From the Laboratory of the Foundation for Chemical Research, Helsinki, Finland.

(*Received April 17th, 1934.*)

THE fermentation of carbohydrates by the root nodule bacteria is a question of great interest, considering that the ability to fix atmospheric nitrogen must necessarily be dependent on the ability to ferment and utilise suitable carbon compounds. The solution of this question might possibly throw some light on the complicated problem of the mechanism of nitrogen fixation.

Although a vigorous fixation of nitrogen occurs when the nodule bacteria are grown in symbiosis with the appropriate host plant, recent investigations have lent additional support to the view that these bacteria are not capable of fixing nitrogen when grown apart from the host. Nor does any fixation occur when the nodules as such are separated from the host plant and cultivated on a synthetic medium, so that the power to fix atmospheric nitrogen cannot be attributable to any special difference in the structure of endonodular bacteria. In our opinion, all this leads to the assumption that the nodule bacteria receive from their host plant some particularly suitable form of carbon nutrition—probably labile carbohydrates, trioses or hexoses—which enables them to carry out the fixation process.

The utilisation of various carbon compounds by the nodule bacteria has been the subject of numerous and extensive investigations. In this respect, our knowledge has been particularly widened by the work of Baldwin and Fred [1927]. These investigations show that nodule bacteria actually utilise a great variety of carbohydrates and related compounds, although different strains differ considerably in this respect.

The decomposition of sugars by the nodule bacteria generally takes place very slowly. Aldohexoses seem to be more readily fermentable than fructose. The characteristics of the fermentation differ considerably for different strains, some strains causing the reaction of the medium to become acid, others alkaline. Very little is known, however, of the fermentation products formed, with the exception of the gum or slime which is obviously produced from carbohydrates.

As far as we know, the actual process of the fermentation of sugars by the nodule bacteria is totally unexplored. This is largely due to the fact that by the old technique hitherto employed—inoculation of the substrate solution with small amounts of the organism in question—the isolation and accurate determination of different fermentation products is scarcely possible owing to the very slow rate of the decomposition. We have therefore used in the present work the technique employed in this laboratory for several years, *i.e.* we have cultivated the organism separately on suitable media and used heavy suspensions

[1] A preliminary note on the subject appeared in the *Acta Chemica Fennica*, B **4**, 62 (1932).

of the cell-mass thus obtained to act upon the substrate. The advantages of this method are obvious. The increased rate of sugar breakdown renders possible the identification and determination of the end-products, whilst the mechanism of the fermentation can be substantially elucidated by interrupting the process at different stages and determining the intermediate products present.

Experimental.

The bacterial species used was a laboratory strain of *Rhizobium trifolii* isolated from red clover and carried in stock for several years.

For the production of bacterial mass, 300–500 slant gelatin test-tubes were used at a time. The nutrient medium was prepared as follows:

Glucose	5·0 g.
Asparagine	2·5 g.
Gelatin	130 g.
Pea-extract	200 ml.
Tap-water	to 1000 ml.

The inoculated cultures were maintained at room temperature for 4 days, after which the bacterial mass was washed off with sterile water. This was done in an inoculation room where all necessary apparatus had been previously sterilised with a Hg-lamp. The suspension was centrifuged clear, and the bacterial mass was washed with warm sterile water. 100 test-tubes produced approximately 1·7 g. of bacterial mass which was practically free from gum.

A total of 20 fermentation experiments was carried out. Calcium lactate was used as substrate in two experiments and calcium butyrate in one, whilst glucose (1–6 g.) was used in all other experiments. In most cases, calcium carbonate was added to neutralise the acids formed during the fermentation. The fermentations were carried out under anaerobic conditions[1] attained by filling the fermentation flasks either with carbon dioxide or nitrogen. All fermentations were carried out at a temperature of 37°. The gases produced were collected and measured over mercury.

The evolution of gas set in, as a rule, about 30 hours after the addition of the bacterial suspension. A considerable portion of the gas was hydrogen.

The destruction of sugar was not complete. In several experiments only about one-half of the glucose originally present was fermented. In very few cases the destruction of sugar amounted to 75 % or more of the original. Sugar determinations were made by Bertrand's method, and the reducing sugar was identified as unfermented glucose by means of its phenylosazone.

After the fermentation had come to an end, the fermented liquid was tested for bacterial purity both microscopically and using gelatin plates. Analyses of the fermentation products were carried out in the manner generally employed in this laboratory.

In one experiment, sterile nodules were used instead of bacterial suspension as the fermenting agent. The nature of the fermentation thereby caused was similar to that caused by the bacterial suspensions.

Results.

It appeared that the relative proportions of fermentation products were closely dependent on the duration of the experiment. During the first stages of the process, considerable amounts of inactive lactic acid were formed from

[1] A preliminary experiment was also made aerobically. The presence of butyric acid in the fermented liquid was noted, but no accurate analysis of the end-products was made.

glucose. Lactic acid is, however, slowly fermented further with the production of butyric acid which is one of the end-products of the fermentation. In addition to lactic and butyric acids, small amounts of acetic acid, ethyl alcohol, carbon dioxide and hydrogen are also formed. The molecular ratio of butyric acid to carbon dioxide and hydrogen is: $C_4H_8O_2:CO_2:H_2=1:2:2$. The fermentation of glucose by the root nodule bacteria is thus a butyric acid fermentation.

Table I shows the results of a series of experiments. In each experiment equal amounts of glucose and $CaCO_3$ and equal amounts of the same bacterial mass were used, the duration of the experiments varying from 90 to 306 hours.

Table I.

Each flask contained 4 g. glucose, 5 g. $CaCO_3$, 2·2 g. moist bacterial mass, 250 ml. tap-water and was filled with nitrogen gas.

Exp. No.	10	11	12
Duration of experiment, hours	90	142	306
Glucose fermented, %	48·2	50·6	84·35
Alcohol, % of fermented glucose	5·25	2·4	6·55
Lactic acid, % of fermented glucose	22·6	18·2	3·0
Butyric acid, % of fermented glucose	24·2	27·3	35·45
Acetic acid, % of fermented glucose	2·9	4·05	Traces

For comparison, results from some other experiments are given in Table II.

Table II.

Exp. No.	7	9	14*
Duration of experiment, hours	96	90	90
Glucose fermented, %	72·2	49·2	29·0
Alcohol, % of fermented glucose	Traces	3·8	Not determined
Lactic acid, % of fermented glucose	40·2	21·4	26·8
Butyric acid, % of fermented glucose	13·0	31·8	30·5
Acetic acid, % of fermented glucose	4·2	Traces	5·1
CO_2, % of fermented glucose	16·0	Not determined	Not determined
H_2, % of fermented glucose	Not determined	Not determined	1·5

* The bacterial mass used in this experiment was somewhat too old and had lost a great part of its activity.

Identification and determination of different fermentation products.

Lactic acid. Quantitative determination of lactic acid was made using the Hirsch-Kauffman [1924] modification of the Fürth-Charnass method.

For identification, lactic acid was isolated from the fermentation liquid as zinc salt after the volatile acids had been distilled off with steam. The product was twice recrystallised from hot water and gave on analysis values consistent with the formula $(C_3H_5O_3)_2Zn, 3H_2O$ for the zinc salt of *dl*-lactic acid. (Found: H_2O, 18·19; ZnO, 26·87 %. $(C_3H_5O_3)_2Zn, 3H_2O$ requires H_2O, 18·18; ZnO, 27·37 %.)

Alcohol was determined according to Kuriloff [1897].

In order to identify the volatile neutral substance produced in the fermentation, the fermented liquid was subjected to steam-distillation at about p_H 8. The distillate was then acidified with sulphuric acid to p_H 2. The volatile substance was again distilled and oxidised with potassium dichromate and sulphuric acid in an autoclave at 110°. The acids thereby formed were determined according to Virtanen [1923] and Virtanen and Pulkki [1928]. It appeared that the oxidation yielded only acetic acid and that, consequently, the volatile fermentation product was ethyl alcohol.

Example. Determination of the original alcohol content according to Kuriloff gave 0·222 g. alcohol in the distillate. From this amount, 46·4 ml. of 0·1 *N* acetic acid were formed on oxidation, corresponding to 0·214 g. ethyl alcohol. The value for the semi-distillation was 36·3 %.

Carbon dioxide. In Exp. 7 the fermentation flask contained:

Glucose	3 g.
$CaCO_3$ (pure)	3·5 g.
Bacterial mass	3·0 g.
CO_2-free tap water	300 ml.

All air in the flask was replaced by CO_2-free nitrogen. The gases evolved were passed into KOH. After completed fermentation the flask was kept in a boiling NaCl solution for 1½ hours, a slow stream of nitrogen being passed through the apparatus. The weight of CO_2 obtained was 0·665 g.

On the basis of the figures presented in Table II the following calculation can be made:

0·091 g. acetic acid	liberates	0·0333 g.	CO_2 from $CaCO_3$
0·871 g. lactic acid	,,	0·213	,,
0·281 g. butyric acid	,,	0·070	,,

Total amount of CO_2 liberated by acids = 0·3163 g.

Assuming that in the production of each molecule of acetic acid one molecule of CO_2 is split off—for 0·091 g. acetic acid 0·0667 g. CO_2—the amount of CO_2 formed in the butyric fermentation will be:

$$0{\cdot}665 - (0{\cdot}3163 + 0{\cdot}0667) = 0{\cdot}282 \text{ g.}$$

In the above experiment 0·281 g. butyric acid was formed, the ratio butyric acid: carbon dioxide being thus 1:2.

Attempts to measure the CO_2 production directly, using strong buffer solutions, failed.

Hydrogen. In Exp. 14, all air was carefully removed from the fermentation apparatus by means of carbon dioxide. The gases produced by fermentation were first led into a mercury burette and thereafter into a burette filled with 40 % KOH. The amount of hydrogen was 162 ml., *i.e.* 13·3 mg. of hydrogen at N.T.P., against 12·0 mg., calculated from the amount of butyric acid formed (0·265 g.).

Succinic and formic acids and glycerol. Succinic acid was determined only in Exp. 1, its amount being found to be 1·2 % of the glucose fermented. This small amount might possibly have been formed from the bacterial mass.

Glycerol is not produced by the nodule bacteria. The same is true of formic acid. Determinations according to Eds [1924–25] gave negative results.

Fermentation of lactic acid by the nodule bacteria.

This was studied in Exps. 6 and 15, using calcium lactate as substrate. It appeared that lactic acid itself is fermented very slowly. Thus, in Exp. 15 only 5 % of the lactic acid was decomposed in 4 days. In Exp. 6, 1·583 g. of pure lactic acid (neutralised with $CaCO_3$) were used together with 1·5 g. of moist bacterial mass. From this amount 1·077 g. were recovered after 11 days. The extent of destruction was thus only 28 %. Amongst the fermentation products were found 0·175 g. of acetic acid and 0·071 g. of butyric acid.

Recovery and identification of unfermented glucose in the fermentation liquid.

Exp. 18. The fermentation flask contained:

Glucose	3 g.
$CaCO_3$	3 g.
Bacterial mass	3·5 g.
Tap-water	250 ml.

The fermentation was carried out in an atmosphere of carbon dioxide. The evolution of gas ceased after 10 days. The volume of the gas was not measured.

The amount of glucose fermented was 2·765 g. or 92·2 %.

After completed fermentation the liquid was centrifuged, acidified with acetic acid and concentrated under reduced pressure to a volume of 95 ml. 30 ml. of the concentrate, containing 68 mg. sugar (as glucose), were heated on the water-bath with some $CaCO_3$ for half an hour. After cooling, 0·2 g. of fresh baker's yeast was added and the flask connected with a 10·0 ml. gas-burette. Temperature 37°. 17·5 ml. of gas were obtained in 23 hours and the fermented liquid did not reduce Fehling's solution. The 68 mg. glucose originally present in the concentrate would have given rise to about 33 mg. or 16·5 ml. of CO_2.

From the remaining 65 ml. of the concentrate the protein substances were removed by $HCl + HgCl_2$, the mercury was precipitated with H_2S and the hydrogen sulphide removed by a stream of air. The solution was then brought with NaOH to about p_H 5 and concentrated *in vacuo* to a volume of 30 ml. and treated with phenylhydrazine hydrochloride and sodium acetate on the water-bath. The phenylosazone formed was washed with hot water and further purified by recrystallisation from 50 % alcohol. It consisted of yellow needles which melted sharply at 205°.

The reducing substance in the fermentation liquid was thus unfermented glucose.

Experiment in which the bacterial mass was grown on liquid medium.

The growth of the nodule bacteria on liquid media is generally very slow, and it is therefore difficult to obtain considerable quantities of the bacteria from liquid cultures. An addition of mould-extract, however, promotes the growth of the nodule bacteria to a surprisingly great extent. The mould-extract was prepared by growing *Aspergillus niger* in suitable media and extracting the mycelium obtained with hot water at p_H 7.

The following experiment was, however, made with bacterial mass grown in an ordinary culture medium of the following composition:

KNO_3	0·5 g.	$FeCl_3$	Traces
K_2HPO_4	0·5 g.	$MnSO_4$	Traces
$MgSO_4, 7H_2O$ ...	0·2 g.	Yeast-extract ...	200·0 ml.
NaCl	0·2 g.	Tap-water ...	to 1000·0 ml.

After incubation for 7 days, the bacterial mass was centrifuged off, washed and suspended in a fermentation flask containing:

Glucose	3 g.
$CaCO_3$	3 g.
Tap-water	250 ml.

The weight of the bacterial mass obtained was 2 g.

The experiment was carried out in an atmosphere of CO_2 for 7 days. The amount of gas produced was 425 ml. The results were:

Glucose fermented ...	1·07 g.	= 35·7 %
Alcohol	0·039 g.	= 3·6 % of ferm. glucose
Butyric acid	0·443 g.	= 41·5 % ,,
Lactic acid	0·058 g.	= 5·5 % ,,

Other products were not determined.

Experiments with sterile nodules.

Twenty-four pea-plants, inoculated with *Rh. leguminosarum*, were grown in six Woulff-flasks in quartz sand under aseptic conditions[1]. After 2 months, the nodules were aseptically cut off from the roots, crushed in a sterile mortar, centrifuged off, washed with sterile water and suspended in a solution containing

Glucose	3 g.
$CaCO_3$	3 g.
Tap-water	250 ml.

The weight of the moist nodule-mass was 5 g. Development of gas ceased after 67 hours. The volume of gas was not measured. Plate culture on gelatin remained clear for over 3 days. Results:

Glucose fermented ...	0·945 g.	= 31·5 %
Alcohol	0·079 g.	= 8·4 % of ferm. glucose
Lactic acid	0·160 g.	= 17·0 % ,,
Acetic acid	0·115 g.	= 12·2 % ,,
Butyric acid	0·185 g.	= 19·6 % ,,

A comparison of the above results with those given in Tables I and II shows that the fermentation caused by separated nodules is, on the whole, very similar to that caused by bacterial suspensions, though in the former case slightly more acetic acid and alcohol were formed.

Catalase content of Rh. trifolii.

Determination of the catalase content of *Rh. trifolii* gave the following results:

$$k = 0{\cdot}00337, \text{ kat. v.} = \frac{0{\cdot}00337}{1350 \times 10^6} = 0{\cdot}0025 \times 10^{-9}.$$

The catalase content of the nodule bacteria is thus comparatively low. For comparison it may be mentioned that Virtanen and Karström [1925] and Virtanen and Winter [1928] found the following values for the catalase content of certain common micro-organisms:

B. fluorescens liquefaciens	$0{\cdot}08 \times 10^{-9}$
B. pyocyaneum	$0{\cdot}41 \times 10^{-9}$
Acetobacter suboxydans	$0{\cdot}01 \times 10^{-9}$
B. coli (10 strains)	$0{\cdot}004$–$0{\cdot}02 \times 10^{-9}$
B. acidi propionici	$2{\cdot}2 \times 10^{-9}$

[1] The method thereby employed has been described by Virtanen *et al.* [1933].

SUMMARY.

Fermentation of glucose by the root nodule bacteria was studied using heavy suspensions of *Rh. trifolii*, grown separately on a pea-extract gelatin medium.

The fermentation thereby caused is of the butyric type. During the early stages of the process considerable amounts of *dl*-lactic acid are formed from glucose. Lactic acid is, however, slowly fermented further with the production of butyric acid which is one of the end-products. In addition to lactic and butyric acids, carbon dioxide, hydrogen and small amounts of acetic acid and ethyl alcohol are also formed. The molecular ratio of butyric acid to carbon dioxide and hydrogen is $C_4H_8O_2 : CO_2 : H_2 = 1 : 2 : 2$.

Similar fermentation of glucose is also caused by crushed nodules from plants grown in sterile quartz sand cultures.

An aqueous extract of mould mycelium was found to stimulate markedly the growth of *Rh. trifolii*.

REFERENCES.

Baldwin and Fred (1927). *Soil Sci.* **24**, 217.
Eds (1924–25). *J. Lab. Clin. Med.* **10**, 1, 59.
Hirsch-Kauffman (1924). *Z. physiol. Chem.* **140**, 25.
Kuriloff (1897). *Ber. deutsch. chem. Ges.* **30**, 741.
Virtanen (1923). *Soc. Scient. Fennica, Comm. Physico-Mathemat.* **1**, 36.
—— v. Hansen and Karström (1933). *Biochem. Z.* **258**, 106.
—— and Karström (1925). *Biochem. Z.* **161**, 9.
—— and Pulkki (1928). *J. Amer. Chem. Soc.* **50**, 3138.
—— and Winter (1928). *Biochem. Z.* **197**, 210.