XIX.—*The Chemical Action of Pure Cultivations of Bacterium Aceti.*

By A. J. Brown.

The following paper contains an account of experiments made for the purpose of investigating some of the chemical actions of a pure cultivation of *Bacterium aceti*.

Pasteur, in his "Mémoire sur la Fermentation Acétique," published in 1864, was the first to show that acetic fermentation is caused by a living micro-organism, which he named *Mycoderma aceti*. He further shows that the acetic acid formed during the fermentation is produced by the oxidation of ethylic alcohol by the oxygen in the air, this action being brought about by the ferment. Moreover, when the fermentation is weakened in certain ways, aldehyde is present, and if the fermentation is allowed to go on after the whole of the alcohol is oxidised, the acetic acid already formed becomes further oxidised to CO_2 and H_2O. Pasteur also believes that succinic acid in small quantity is always formed as a bye-product during the acetic fermentation.

E. Wurm (*Dingl. polyt. J.*, **235**, 225) investigated the acetic fermentation, and confirms Pasteur's statement that the formation of vinegar from alcoholic liquids is due to an organised ferment.

In the *Comptes rendus* of July, 1880, p. 236, Boutroux describes an action of the acetic ferment on glucose, but this paper will be more fully referred to later on.

Cohn (*Biol. d. Pflanzen*, **2**, 173), and more particularly Hansen (*Meddelser fra Carlsberg-Laboratoriet*), have investigated the acetic ferment (*B. aceti*) morphologically, but do not appear to have studied its chemical action.

Before describing my experiments, I think it advisable in a research of this nature to describe the method of working, and the means used for the purification of the ferment.

As *B. aceti* is strictly an aërobic ferment, and consequently as my experiments required free access of air, the method of working with flasks or test-tubes plugged with sterilised cotton-wool was found very suitable. All the vessels and cotton-wool used, and also the various liquids in which the ferment was grown, were sterilised with all the precautions advised by Klein ("Micro-organisms and Disease," *Practitioner*, 1884). No solution was considered sterilised and fit for use unless it had kept quite free from all forms of living organisms for at least a week at a temperature of 28°. Inoculation from one vessel to another was effected by means of a capillary glass tube freshly drawn out and pointed at one end, so that it could be forced through the cotton-wool plugs in the necks of the vessels, and each tube was not used more than once. The cotton-wool plugs of the vessels were kept covered with sterilised paper, and, before inoculating from one vessel to another, the surface of the plug was singed by a gas flame.

I found the most suitable method for obtaining a pure culture of *B. aceti* was a combination of Kleb's "Fractional" and v. Nägeli's "Dilution" methods. Inoculation in gelatin media did not give such satisfactory results, because *B. aceti* being so strictly aërobic it only grows on the immediate surface of the gelatin, and in such an attenuated film that it is difficult to detect it in its early stage of growth before it has spread over a considerable surface.

The *B. aceti* used in my experiments was originally taken from the surface of a beer that had been kept exposed to the air in a warm place until the acetic fermentation developed. This ferment was inoculated into the first of a series of 10 test-tubes, containing a sterilised solution of 2 per cent. ethylic alcohol in yeast water;* the inoculations being made from one tube to another as soon as a visible growth was observed (generally from 36 to 48 hours). Finally, the culture was further tested by the "dilution" method, and showed its purity by always producing a growth of *B. aceti* (or no growth at all) in the tubes into which the diluted ferment was inoculated. Further, if the reactions of this ferment, which I describe further on, are considered, the comparative simplicity of the action in some cases,

* Used by Pasteur, and prepared by boiling 7 per cent. yeast, pressed as dry as possible, in water, and filtering until quite bright.

and the want of action altogether in others, will leave no doubt that my experiments were made with a pure cultivation of acetic ferment. *B. aceti* has so many different forms of growth, that I do not consider the microscope alone gives much trustworthy aid in detecting foreign ferments mixed with it.

The mode of growth of pure *B. aceti* in alcoholic or sugar solutions, is in the form of a rather greasy pellicle covering the surface of the liquid, and inclined in its young stage of growth to creep up the moist sides of the containing vessel.

This pellicle varies in thickness from an almost invisible film on the surface of such a mixture as dextrose and Pasteur's solution, to the thickness of stout paper on diluted claret. Slight agitation easily breaks the surface of this pellicle, and, after its somewhat greasy surface is wet, it sinks to the bottom of the liquid, only, however, for another growth to form again on the surface, if the liquid still contains the proper nourishment for it. The liquid below the pellicle is usually turbid with isolated cells of the ferment, and, after a cultivation has been allowed to remain undisturbed for some weeks, a considerable deposit of the growth is found at the bottom. *B. aceti* is, strictly speaking, an aërobic ferment; if inoculated into solutions kept free from oxygen, it will not increase or produce any chemical changes in the liquid, but, nevertheless, is capable of living under these circumstances for a long time. I have kept an inoculated solution out of contact with air for six months without its showing any signs of growth, but on admitting filtered air a strong growth started in a few days.

Hitherto, two species of bacteria said to have the power of oxidising alcohol to acetic acid have been described, viz., the ordinary *B. aceti* with which we are now concerned, and the *B. Pasteurianum* of Hansen. There is, however, at least one other distinct species, about which I hope to say something in another communication. Under these circumstances it seems advisable to describe shortly the morphology of the species with which I have worked (viz., *B. aceti*).

B. aceti in its normal state, freshly growing as a pellicle on the surface of a liquid, appears under the microscope as a mass of cells about 2μ* in length, and slightly contracted in the middle, giving them a sort of figure of 8 appearance. These cells are united into chains of variable length, which are easily broken up by pressure of the cover-glass. Frequently the cells are quite divided in the middle, thus producing strings of micrococcus-like forms; both forms being sometimes found in the same chain. The above two forms are those

* No exact measurements of *B. aceti* can be given, as all the forms differ very much amongst themselves in size, varying with the different culture fluids, amount of acid, &c.

usually present when the ferment is growing vigorously at the surface of a liquid. But in the liquid below the surface film, and on the bottom of the containing vessel, abnormal forms are often found differing very much from the ordinary surface growth: this is more especially the case in old cultivations. These forms often attain the length of 10—15μ, or even more; in some cases their form is that of leptothrix threads of even thickness throughout their length; in others, the long cells are swollen out in two or three places along their length, giving them a most irregular appearance. These cells are generally of a dark-grey colour. At their ends a short chain of short rods or micrococci is sometimes observed. The other forms most frequently seen are short rods about 3μ in length, and micrococci about 1μ in diameter, floating freely in the culture liquid. My observations on the morphology of *B. aceti* agree very well with the drawings given by Hansen (*Meddelser fra Carlsberg-Laboratoriet*, **2**, 1879), and by Zopf (*Die Spaltpilze*, 1885, pp. 9 and 63).

The shorter rods and cells of *B. aceti*, when floating freely in culture fluid, are motile, but I have not noticed this property in the large abnormal forms.

When treated with dilute solution of iodine, *B. aceti* is stained yellow. Hansen states that the only difference between this ferment and *B. Pasteurianum* is that the latter is stained blue by similar treatment.

Boiling with dilute caustic potash quickly disintegrates the pellicle of *B. aceti*; also on treating it with strong sulphuric acid it is at once broken up, and the further addition of iodine merely turns it yellow.

Oxidation of Ethylic Alcohol.

Klein ("Micro-organisms and Disease," 1884), when describing *B. aceti*, states that "pure cultivations have not been made with it, and before deciding whether it is the real cause of the acetic acid fermentation, experiments with such pure cultures, *i.e.*, inoculations of alcoholic fluids with it, are required." For this reason, the first experiment I describe concerns this well-known action.

A litre flask half full of a 5 per cent. solution of pure ethylic alcohol in yeast water, and quite free from acid, was sterilised by the method described above. It was afterwards kept for 10 days at a temperature of 28° to be quite sure of its sterility. This being ascertained, the solution was inoculated with a trace of the pure cultivation of *B. aceti*. The flask was then placed on a hot water tray connected with a thermostat, and there kept at a constant temperature of 28°. On the second day after inoculation, a fine film of ferment had com-

menced to grow on the surface of the solution, which film increased in substance slightly during the next few days. On the tenth day, the flask was opened and found to smell strongly of acetic acid. 100 c.c. of the solution was distilled, pure water being frequently added to the retort, until the whole of the volatile acid had passed over. This volatile acid on titration gave a percentage of 1·021 acid calculated as acetic acid on the original 100 c.c. of solution distilled. The residue in the retort showed only 0·006 per cent. of non-volatile acid. There has been formed, therefore, during the growth of *B. aceti* in the original alcoholic solution, 1·021 per cent. of volatile acid and a mere trace of non-volatile acid. In order to ascertain the nature of the volatile acid, the rest of the solution was carefully distilled, and the very acid distillate neutralised with an excess of pure baric carbonate. After filtration, the whole of the solution was evaporated to dryness, and the residual salt dried at 130°. A weighed portion of the salt was decomposed in a platinum crucible by sulphuric acid, ignited, and the residual baric sulphate again weighed; on calculating, the following result was obtained:—

Per cent. of Ba in salt	53·65
„ of Ba in $Ba(C_2H_3O_2)_2$	53·73

showing conclusively that acetic acid was the one and only volatile acid formed in the above experiment with a pure cultivation of *B. aceti*.

The trace of non-volatile acid formed in the experiment seemed to answer to the tests for succinic acid, thus agreeing with Pasteur's observations; but the quantity found was too small for satisfactory identification. Traces of a body resembling aldehyde are generally found on distilling an acetic fermentation of *B. aceti*; this is more especially the case when the ferment is growing with an insufficient supply of oxygen.

Pasteur stated in his "Mémoire" that acetic acid is completely burnt to carbonic acid and water by *B. aceti*, when there is no alcohol in the solution. I confirmed this by the following experiment. A solution of 0·75 per cent. acetic acid in yeast water, sterilised as usual, was inoculated with *B. aceti*. The ferment grew with difficulty, and only formed an extremely thin pellicle on the surface of the liquid. After six weeks, the flask was opened and the acid determined. Only 0·25 per cent. of acid remained, showing that 0·5 per cent. of acid had disappeared during the growth of the ferment. A second flask, not inoculated, was found during the same time not to have lost any appreciable amount of acid. If an alcoholic solution is allowed to ferment until the whole of the alcohol is converted into acetic acid, I find that the acid so formed is much more quickly decomposed by

the ferment than in the experiment I have just described; this most probably is due to the strong pellicle of ferment formed during the time the oxidation of the alcohol was going on.

Action of B. aceti on Propylic Alcohol.

Seeing that ethylic alcohol is so freely oxidised by *B. aceti*, it appeared desirable to ascertain whether this action did not extend to the other alcohols of the same series. I first experimented on normal propylic alcohol, and prepared a solution containing 3 per cent. of the pure alcohol in yeast water. After sterilising, &c., in the usual way the solution was inoculated with *B. aceti*. For the first few days the ferment grew very slowly, but later a fine pellicle was developed on the surface. The flask was opened 14 days after inoculation, when the liquid was found to have a strongly acid odour. A sample taken for estimation of the total acid gave 1·20 per cent. calculated as $CH_3 \cdot COOH$. The rest of the solution was distilled, and the acid distillate neutralised with carbonate of barium, filtered and evaporated to dryness. After drying at 130°, a weighed portion of the salt was decomposed with sulphuric acid, ignited, and weighed. On calculation, the following result was obtained:—

Per cent. of Ba in salt	48·35
„ of Ba in barium propionate	48·41

showing that normal propylic alcohol is oxidised to propionic acid by the action of *B. aceti*, just as ethylic alcohol is oxidised to acetic acid. But a mere trace of non-volatile acid was found in the original solution after distilling off the propionic acid.

Action of B. aceti on other Alcohols.

The next experiments were made with methylic alcohol. I found this alcohol had to be purified by repeated distillation to get rid of all traces of resinous matter before the ferment would grow freely in its presence.

A 1 per cent. solution of this in yeast water was inoculated with *B. aceti*. In a few days the ferment was growing freely. On opening the flask after three weeks, the solution was found slightly *alkaline*. (Before going further I had better add that *B. aceti* grows freely in yeast water alone, the reaction becoming slightly alkaline. On distilling this solution, ammonia or ammonium carbonate distils over. The residue, after slightly acidifying with a few drops of sulphuric acid, yields a very small quantity of volatile acids which have a slight reducing action on silver nitrate and permanganate, and

which are probably a mixture of butyric with a little formic acid.) The alkaline distillate on examination contained only ammonia and methylic alcohol. The residue in the flask, after slightly acidifying with a few drops of sulphuric acid, was again distilled. A very small quantity of volatile acid was obtained, which had a slight reducing action on $AgNO_3$ and on $HgCl_2$, but the very small quantity of the acid and also its character is only what may be obtained from a fermentation of yeast water alone.

Another experiment was made on a solution of yeast water containing 1 per cent. of methylic alcohol and some calcic carbonate. This fermentation was allowed to go on for four weeks, during which time the ferment grew very freely. On opening the flask and distilling, after slightly acidifying with sulphuric acid, only 0·008 per cent. volatile acid was found. From the above two experiments, it is evident that *B. aceti* is unable to oxidise methylic alcohol to formic acid, under circumstances in which it acts freely on ethylic and propylic alcohols. But as it was possible that methylic alcohol might have been oxidised directly to carbonic acid and water in the experiments quoted, a flask containing 500 c.c. of yeast water was sterilised, and afterwards exactly 5 c.c. of methylic alcohol, sp. gr. 0·8151, was added, and the solution inoculated with *B. aceti*. In order that the alcohol in the solution should not be lost by diffusion of its vapour through the cotton-wool plug, the mouth of the flask was closed by an india-rubber stopper pierced by two tubes, so arranged that filtered pure air could be drawn through the flask every second day, the air that came from the flask being passed through a chloride of calcium tube to arrest any methyl alcohol that might be with it. The ferment in this experiment grew with great freedom, and the fermentation was allowed to go on for four weeks and a half. On opening the flask, the solution was carefully distilled until all the alcohol had passed over, and the sp. gr. of the distillates was taken. On comparing these weights with a table, I found 4·90 c.c. of alcohol had distilled over, against the original 5·00 c.c. with which the experiment had been started. These results agree as closely as could be expected considering the method used, and leave no doubt that, under the circumstances of my experiment, methylic alcohol is not acted on in any way by *B. aceti*. Why this should be so with an easily oxidisable liquid like methylic alcohol, when ethylic and propylic alcohols are acted on so readily, it is difficult to say. It can hardly be because the products of the action of *B. aceti* on methylic alcohol are poisonous to itself, for if so, why should it thrive so well in the presence of the alcohol?

I have made repeated attempts to oxidise isoprimary butylic

alcohol (b. p. 108°) by means of *B. aceti*, but have been unsuccessful, although the ferment will grow feebly in 0·5 per cent. solution of this alcohol in yeast water.

I have not succeeded in making *B. aceti* grow at all in presence of amylic alcohol (fermentation alcohol).

Action of B. aceti on the Carbohydrates.

In the *Comptes rendus* of March, 1878, p. 605, is a paper by Boutroux entitled "Sur la fermentation lactique," in which the author describes, as a continuation of Pasteur's work on the lactic ferment (*Ann. Chim. Phys.*, 1857), a purified cultivation of this ferment, which grows as a pellicle on the surface of solutions containing sugar and nitrogenous matter, and had the power of converting this sugar into lactic acid. He further finds that this ferment has the power of converting alcohol into acetic acid, and concludes that the lactic and acetic ferment are one and the same organism.

In the *Comptes rendus* of July, 1880, p. 236, another paper from Boutroux appears correcting his former conclusions, and stating that the acid formed by his ferment from sugar is not lactic but gluconic acid. He gives analyses of the acid and its salts, and further states that the ferment which forms gluconic acid is *Mycoderma aceti*, and that he was in error in calling it lactic ferment.

It appeared desirable to repeat this experiment with my pure cultivation of *B. aceti*. Boutroux in his experiments grew the ferment in a solution of glucose in yeast water containing a quantity of calcic carbonate to neutralise the acid when formed ;* but as there is in this case a considerable quantity of organic matter from the yeast present which might possibly complicate the reaction, I preferred to make my experiments with solutions containing merely dextrose, and inorganic salts of known composition to serve as food for the ferment. For this purpose, I prepared a flask containing 3 litres of a solution composed of 2 per cent. dextrose dissolved in Pasteur's mineral solution, and to this 10 grams of pure calcic carbonate was added. This solution was sterilised, and after being kept as usual was inoculated with *B. aceti* on August 2nd. The ferment grew more slowly than in yeast water and dextrose, and the pellicle formed on the surface was extremely thin. On September 10th, most of the calcic carbonate having been dissolved, the flask was opened for examination.

* This acid fermentation of dextrose goes on freely in a solution without $CaCO_3$ being added; but when about 0·4 per cent. acid (calculated as $CH_3{\cdot}COOH$) is formed, further action is much retarded.

A portion of the solution was distilled and examined for ethylic alcohol, but none was found. The residue in the retort was then rendered slightly acid with sulphuric acid, and the solution again distilled. The distillate gave no acid reaction, showing the absence of acetic and other volatile acids.

The bulk of the original solution was then filtered as clear as possible, and evaporated slowly to a small bulk. To this solution, a large excess of alcohol of 0·83 was added, which produced a bulky precipitate of a brownish gummy nature. After allowing the whole to stand 24 hours, the bright alcoholic solution was poured off, and a small quantity of water added to the precipitate, which dissolved it completely to a dark brown solution. After decolorising with animal charcoal, a large excess of alcohol was again added; this brought down the precipitate in nearly white flocks. The solution was allowed to stand until quite bright, and then filtered. After washing the precipitate thoroughly with alcohol and well draining, the salt was again dissolved in water, and the solution heated to 100°. Boiling alcohol was then added so as just to produce a slight permanent milkiness, and the solution was put on one side to cool slowly. After 48 hours, the bottom and sides of the beaker were covered with beautifully white roundish concretions, evidently of a crystalline nature. Under the microscope they were found to be masses of minute acicular crystals.

After drying this salt at 100°, two estimations of the CaO in it were made, giving—

No. I. 12·63 per cent. CaO.
No. II. 12·68 „ „

Calcic gluconate, according to Hertzfeld's formula (*Annalen*, **220**, 335), $(C_6H_{11}O_7)_2Ca + 1OH_2$, contains 12·50 per cent. CaO.

0·6454 gram of this salt was burnt with lead chromate and potassic dichromate. This combustion gave 0·3122 gram CO_2 and 0·7535 gram OH_2. On calculation, the Ca being taken as the mean of my two analyses, we get the following result:—

	Found.	$(C_6H_{11}O_7)_2Ca + 1OH_2$.
C	31·84	32·14
H	5·37	5·36
O	53·75	53·57
Ca.....	9·04	8·93

The above analyses leave no doubt that the salt with which we are dealing is calcic gluconate.

This salt does not reduce Fehling's solution, neither has its solution any action on polarised light. It reduces silver nitrate with

great ease, and also prevents the precipitation of ferric oxide by ammonia. Its aqueous solution, on being freely exposed to the air for several weeks, gradually deposits the crystalline form of the salt again, unchanged. On heating the dry salt strongly, it intumesces in a remarkable manner, and finally burns to a white ash. I have prepared the free acid from the calcic salt; and the solution of the free acid when evaporated in a vacuum leaves it as an uncrystallisable, glassy, nearly colourless mass. Its aqueous solution is intensely acid to the taste when concentrated, and on heating, even below 100°, it begins to turn brown and decompose.

In my fermentation experiment with dextrose, calcic gluconate appeared to be the only soluble calcic salt present. I therefore examined the insoluble residue from the fermentation, but found it to consist of calcic carbonate and cells of *B. aceti* alone. It therefore appears that the sole product of the action of *B. aceti* on dextrose is gluconic acid. If we consider dextrose, as it is generally taken, to have the constitution of an aldehyde of the hexhydric alcohol, mannitol, the reaction would probably be represented thus:—

Dextrose.		Gluconic acid.
$CH_2{\cdot}OH$		$CH_2{\cdot}OH$
$CH{\cdot}OH$		$CH{\cdot}OH$
$CH{\cdot}OH$		$CH{\cdot}OH$
$CH{\cdot}OH$	$+ O =$	$CH{\cdot}OH$
$CH{\cdot}OH$		$CH{\cdot}OH$
COH		$COOH$

Gluconic acid being the sole product of the oxidising action of *B. aceti* on dextrose, considerably strengthens the idea of the constitution of dextrose being partly aldehydic, as represented in the above formula.

B. aceti on Sucrose.

My next experiments were made to ascertain what effect *B. aceti* might have on solutions of cane-sugar or sucrose. Having failed to make the ferment grow in solutions of cane-sugar and Pasteur's mineral medium, I prepared and sterilised a solution of yeast water containing 4 per cent. of cane-sugar, and inoculated it with *B. aceti* as usual. In a few days, the ferment was growing strongly. After six weeks, the flask was opened and the solution examined. It was found to be perfectly free from acid, and did not reduce Fehling's solution.

Another experiment was made in which the polarising power of the solution was noted previous to inoculation. After *B. aceti* had grown freely in the solution for three weeks, the flask was opened. The

contents were carefully made up to the original volume and filtered. On examining the solution with the polariscope, the original angle was obtained. A blank experiment made with the same yeast water and inoculated at the same time, showed that both before and after the growth of *B. aceti* there was no action on polarised light. The cane-sugar solution in my second experiment had a slight alkaline reaction, and did not reduce Fehling's solution; but on treating it with dilute sulphuric acid and warming, it reduced freely.

The above experiments show that *B. aceti* is unable to break up or change the molecule of cane-sugar.

Now if the constitution of cane-sugar is really aldehydic, as it is usually represented in the formula $O\begin{cases} C_5H_6(OH)_4\cdot COH \\ C_5H_6(OH)_4\cdot COH \end{cases}$, it appears strange that *B. aceti* is unable to attack the aldehyde part of the molecule in this case, in the same way that it acts upon dextrose when oxidising it to gluconic acid.

Action of B. aceti on Mannitol.

After my previous experiments, it appeared very desirable to study the action of the ferment upon mannitol, the alcohol corresponding to the aldehyde dextrose on which the ferment acts so freely.

Gorup-Besanez (*Ann. Pharm.*, **118**, 273), on oxidising mannitol by means of platinum black, found that mannitic acid, mannitose (a fermentable sugar), and an unfermentable gummy substance were the chief products. Mannitic acid he analysed and described, but he was unable to separate mannitose from the gummy product. He describes mannitose as not producing the least deflection of the plane of polarisation, but as reducing Fehling's solution freely.

Berthelot (*Ann. Chim. Phys.*, **50**, 369) states that by fermenting a 10 per cent. solution of mannitol in water, in contact with certain animal membranes, a "glucose" is sometimes formed. The results are very irregular, the "glucose" found varying from a mere trace to a tenth of the amount of mannitol used, the highest result obtained. The "glucose" could not be obtained pure, but Berthelot describes it as uncrystallisable, very soluble in alcohol, fermentable, as reducing Fehling's solution, and probably lævorotatory. The animal membrane used in the experiment is thought to be the cause of conversion of mannitol into sugar (?).

A. Fitz (*Ber.*, **9**, 1352) states that during the schizomycetic fermentation of mannitol, normal butyl and ethyl alcohols, and butyric and lactic acids are formed.

Hecht and Iwig (*Ber.*, **14**, 1760), by the oxidation of mannitol by alkaline potassium permanganate, obtained formic, oxalic, and a small

quantity of tartaric acid, and also a sugar which reduces Fehling's solution, and is probably mannitose.

F. Dafert (*Ber.*, **17**, 227) states that the products of the oxidation of mannitol vary according to the agent used, and also the time and temperature of oxidation. Carbonic anhydride, water, formic, mannitic, saccharic, tartaric, and probably glycollic acids have been observed; also a mannitose-like substance, and a sugar, mannitose, are invariably formed. Mannitose reduces Fehling's solution, and is optically inactive.

My first experiment on the oxidising action of *B. aceti* upon mannitol was made with a 2 per cent. solution of the latter body (Pasteur's mineral medium and a little gelatin being added as food for the ferment). After inoculation with *B. aceti*, the ferment grew freely. In six weeks' time, the fermented solution was examined. No acid had been formed, but the solution had acquired a very sweet taste and reduced cupric oxide freely, a result which pointed to some sugar having been formed from the mannitol during fermentation.

In order to separate this substance, the solution was evaporated on a water-bath and the residue exhausted with boiling alcohol, sp. gr. 830. The part undissolved by the alcohol consisted chiefly of gelatin, and possessed no cupric oxide reducing power. The hot alcoholic solution (which reduced Fehling's solution freely), on cooling, deposited some crystals of unaltered mannitol; these were filtered off, and the clear solution evaporated. A brown, very sweet syrup was left, which was treated with hot absolute alcohol, in which it dissolved completely, but on cooling a trace of mannitol and a little gummy matter were deposited. The clear solution was separated from this deposit and evaporated. After completely expelling all the alcohol from the syrupy residue, it was dissolved in water and decolorised by animal charcoal. A colourless, very sweet solution was thus obtained. On examining with the polariscope, the matter in solution was found to possess an $[\alpha]_j = -72{\cdot}0$, whilst the cupric oxide reducing power was found to equal $\kappa_{386}75{\cdot}23$. On adding a little yeast to the solution, it fermented slowly, and in a few days all its optical activity had disappeared, but the unfermented residue still reduced Fehling's solution slightly.

It is evident from the above experiment that during the growth of *B. aceti* in a solution of mannitol, a fermentable sugar is formed, possessing a high lævorotatory power together with a high cupric oxide reducing power. Lævulose is the only sugar at present known that possesses these properties.

In order to study this interesting reaction more closely, a solution was prepared containing 25 grams of mannitol in 1 litre of yeast water. After sterilisation and inoculation with *B. aceti* as usual, the

fermentation appeared to go on more briskly than in my first experiment. At the end of five weeks, when the flask was opened for examination, the solution contained no free acid. After evaporating the solution to a syrup, at a temperature of 70°, it was treated with alcohol as in my first experiment. No mannitol, however, was found, showing that the whole 25 grams had been decomposed during the fermentation. The brown syrup, purified as in my first experiment by absolute alcohol, was further treated with cold methylic alcohol, sp. gr. 816. In this, however, it was completely soluble. After evaporating this solution to expel the alcohol, the syrup remaining was dissolved in water and decolorised by animal charcoal as before. The optical activity and the cupric oxide reducing power were then determined with the following result :—

$$[\alpha]_{j386} = -86{\cdot}07$$
$$\kappa_{386} = 86{\cdot}11$$

In this experiment, therefore, the proportion of the rotatory power to the cupric oxide reducing power is much the same as in my first experiment, but the actual amount of lævorotary sugar is much greater.

In order to see if the sugar (presumably lævulose) which we have here could be further purified, a solution of the syrup was made in alcohol 820 sp. gr., and then excess of ether was added so as to fractionally precipitate the substances in solution. Three fractions were thus obtained, and the cupric oxide reducing power and optical activity were determined in each, with the following results :—

1st Fraction... $[\alpha]_{j386} = -83{\cdot}6$, $\kappa_{386} = 87{\cdot}3$

2nd Fraction .. $[\alpha]_{j386} = -89{\cdot}98$, $\kappa_{386} = 88{\cdot}86$

3rd Fraction... $[\alpha]_{j386} = -97{\cdot}49$, $\kappa_{386} = 97{\cdot}35$ (?).

These experiments point very strongly to the conclusion that the sugar that has been formed from mannitol is *lævulose*, the last fraction especially having a rotatory power closely approximating to the supposed rotatory power of pure lævulose, viz., $[\alpha]_j - 106$.

I next endeavoured to ascertain the true rotatory power and cupric oxide reducing power of the lævulose by means of a fermentation experiment. The cupric oxide reducing power and rotatory power of a solution of the sugar of known specific gravity were carefully determined and the solution was then fermented, precautions being taken to prevent loss of alcohol. After fermentation, the alcohol was distilled, and the proportion of sugar decomposed was calculated from the alcohol found (Pasteur's figure 48·5 grams alcohol = 100 grams

$C_6H_{12}O_6$ sugar being used in this calculation). After determining the rotatory power and reducing power of the residue from the fermentation, the $[\alpha]_{j386}$ and κ_{386} of the fermented sugar were calculated from the figures thus obtained, with the following result:—

$$[\alpha]_{j386} = -99{\cdot}8 \text{ at } 15{\cdot}5^\circ \text{ C.}$$
$$\kappa_{386} = 94{\cdot}26$$

Owing to at least one doubtful factor having to be used in the above calculation (viz., the proportion of alcohol equal to sugar fermented, which is unknown for lævulose), the figures just given cannot be considered to represent with great accuracy the $[\alpha]_j$ and κ of the lævulose from mannitol. Although these figures differ slightly, therefore, from those usually assigned to the lævulose from invert sugar (viz., $[\alpha]_j - 106$ and $\kappa = 100$), there is little or no doubt that the two lævuloses are identical.

The following properties of the lævulose from mannitol, which agree exactly with the properties of lævulose both from inulin and invert sugar, most strongly confirm this idea.

At ordinary temperatures, the sugar is a colourless, very sweet syrup, quickly turning brown when heated at 100°. It is slightly soluble in absolute alcohol in the cold, but on heating dissolves freely. On cooling the solution so formed, the sugar again falls out as a syrup; on further cooling below 0°, the syrup solidifies and becomes opaque, but so far I have not been able to obtain crystals of either this sugar or the sugar from inulin, after the manner described by Jungfleisch and Lefranc (*Compt. rend.*, **93**, 547). On treating an aqueous solution of the lævulose from mannitol with calcic hydrate, according to Dubrunfaut's process, a pasty mass of microscopic crystals of a lime salt is formed resembling exactly that obtained with the lævulose of invert sugar.*

After completely fermenting aqueous solutions of the lævulose from mannitol, there is always a small unfermented residue left which has no perceptible action on polarised light, but reduces cupric oxide. An estimation of its reducing power after allowing for products of fermentation left in solution, gave $\kappa_{386} = 33{\cdot}4$. The proportion of this compound to the lævulose in the syrup from a mannitol fermentation varies, but is generally about 15 per cent. How far it has been formed during the original fermentation, or how far from the decomposition of lævulose during evaporation of solutions, &c., is doubtful.

The experiments I have described show that mannitol is completely oxidised by *B. aceti*, and that the main product formed is lævulose.

* Since writing the above, I have converted lævulose from mannitol into mannitol again by means of sodium amalgam. Lævulose both from inulin and from invert sugar is converted in a similar manner into mannitol.

In its action upon mannitol, therefore, *B. aceti* behaves differently from all other oxidising agents which have been described (see above). Previous investigators have always found a small quantity of a sugar amongst the products of oxidation of mannitol, but this sugar is an optically inactive one, mannitose.

If the constitution of lævulose be represented by the formula which Kiliani (*Ber.*, **18**, 3066) has recently shown to be probably the correct one, the following will perhaps represent the action of *B. aceti* upon mannitol :—

$$\begin{matrix}\begin{Bmatrix}CH_2\cdot OH\\CH\cdot OH\\CH\cdot OH\\CH\cdot OH\\CH\cdot OH\\CH_2\cdot OH\end{Bmatrix}\\ \text{Mannitol.}\end{matrix} + O = \begin{matrix}\begin{Bmatrix}CH_2\cdot OH\\CH\cdot OH\\CO\\CH\cdot OH\\CH\cdot OH\\CH_2\cdot OH\end{Bmatrix}\\ \text{Lævulose.}\end{matrix} + OH_2.$$

In my experiments, one fact is very noticeable, viz., that no acid is formed during the decomposition of mannitol. Fermentations left for a month after the complete disappearance of all the mannitol showed no trace of acid. From this it was evident that the lævulose formed could not be further oxidised by the ferment to gluconic acid in the same way as dextrose. Experiments made by growing *B. aceti* in solutions containing lævulose prepared from inulin showed that this sugar also was not oxidised by the ferment. This in itself is evidence that there must be a very considerable difference between the molecular constitution of lævulose and dextrose, and appears to strengthen the theory that the one is a ketonic compound, and the other aldehydic. Thus,

$$\text{Lævulose..}\begin{Bmatrix}CH_2\cdot OH\\CH\cdot OH\\CO\\CH\cdot OH\\CH\cdot OH\\CH_2\cdot OH\end{Bmatrix}\text{Not oxidised by } \textit{B. aceti}.$$

$$\text{Dextrose}\begin{Bmatrix}CH_2\cdot OH\\CH\cdot OH\\CH\cdot OH\\CH\cdot OH\\CH\cdot OH\\COH\end{Bmatrix}\text{Oxidised by } \textit{B. aceti} \text{ to } \begin{Bmatrix}CH_2\cdot OH\\CH\cdot OH\\CH\cdot OH\\CH\cdot OH\\CH\cdot OH\\COOH\end{Bmatrix}\text{Gluconic acid.}$$

As we know now that by means of *B. aceti* we can convert mannitol into lævulose, it follows that dextrose can be converted into lævulose through this reaction, by first transforming it into mannitol by means

of sodium amalgam, *always supposing that the mannitol so formed is identical with that from manna.*

I think the experiments just described will be of interest to biologists, as well as chemists, as they help to show that the vital functions of certain organised ferments are most intimately connected with the molecular constitution of bodies upon which they act.

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