The Bacterial Production of Acetylmethylcarbinol and 2.3-Butylene Glycol from Various Substances.—II.*

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The action of *B. subtilis* (Cohn), *B. mesentericus vulgatus* (Flügge) (*B. vulgatus* (Flügge) Migula), and *Tyrothrix tenuis* (Duclaux) (*B. tenuis* (Duclaux) L. and N.) on various substances has been investigated by Péré (1). This observer, on distilling his various culture media, obtained lævo-rotatory distillates strongly reducing to Fehling's solution. In all cases he concluded that the volatile substance present was glyceraldehyde, and upon his results based a theory that sugars undergoing bacterial fermentation break down primarily to a triose, that is to say, glycerose.

He was, however, unable to characterise his compound satisfactorily; for example, it did not give Schiff's reaction, no osazone was obtainable, and although in some cases he obtained small quantities of lead, calcium, and barium salts of an acid formed by the oxidation of his volatile substance with nitric acid, which he took for salts of glyceric acid, the quantities analysed were so small that no reliance can be placed upon the results. Moreover, none of his salts was obtained in crystalline form.

Soon after the appearance of Péré's work Wohl (2) succeeded in preparing glyceraldehyde in a pure state, and found that it was non-volatile in steam, that it gave Schiff's reaction, and also formed a highly characteristic osazone, M.P. 131° C. It seemed therefore impossible that the volatile substance obtained by Péré could have been glyceraldehyde, and a further investigation of the subject has therefore been made.

In a previous communication (3) the action of *B. lactis aërogenes* and *B. cloacæ* on many carbohydrates, alcohols, etc., has been described. With glycerol itself the liquid obtained on distilling the culture medium was absolutely without reducing power. In the majority of cases, however, the distillate possessed reducing power which was shown to be due not to the presence of glyceraldehyde, but to that of acetylmethylcarbinol, $CH_3{\cdot}CH(OH){\cdot}CO{\cdot}CH_3$, the substance responsible for the Voges and Proskauer reaction (4), which had previously been observed as a product of the bacterial fermentation of glucose by Grimbert (5). The above experiments were carried out under anaërobic conditions, but Walpole (6) has shown that

* For first part see paper read February 1, B, vol. 84. p. 492.

the yield of carbinol may be increased by aërobic culture. The whole of Péré's experiments were carried out under aërobic conditions, and although he employed different organisms from those given above, they have all been shown by Desmots (7) to be capable of producing acetylmethylcarbinol under suitable circumstances. Desmots, in fact, describes experiments very similar to those carried out by Péré, but makes no mention of the possible formation of glyceraldehyde. It seems therefore not at all unlikely that Péré's volatile reducing substance was acetylmethylcarbinol and not glyceraldehyde. His experiments have accordingly been repeated, using identical culture media and conditions of growth, and the results of these investigations form the subject of the present communication. In addition to repeating Péré's experiments a quantitative estimation of the action of *B. lactis aërogenes* on glycerol has been made. In this case neither acetylmethylcarbinol nor glyceraldehyde is obtained.

Action of B. subtilis *and* B. mesentericus vulgatus *on Mannitol and* Tyrothrix tenuis *on Glucose.*

The culture media were made up exactly in the same way as those used by Péré, and in the case of the first two organisms named consisted of 20 grm. of mannitol in 200 c.c. water containing 2 grm. ammonium phosphate, 1 grm. ammonium sulphate, and 0·4 grm. potassium phosphate. For the experiment with *Tyrothrix tenuis* 5 grm. of glucose were made up to 100 c.c. with broth. After sterilisation and inoculation with the organism in question, the various culture media were incubated at 37° C. In every case growth was continued for 30 days, after which time the cultures were worked up according to Péré's directions. To take one example—the action of *B. subtilis* on mannitol—after the 30 days' incubation the culture medium was made acid with citric acid and distilled, the distillate was found to be strongly reducing and lævo-rotatory, and also gave a very strong Voges and Proskauer reaction, which is characteristic of acetylmethylcarbinol, but is not given by glyceraldehyde. The remaining distillate was then made alkaline and again distilled, yielding a second time a reducing lævo-rotatory body giving the Voges and Proskauer reaction. This second distillate was then steam-distilled for three hours, and by the end of that time the whole of the reducing body had passed over with the steam, the residue being non-reducing and optically inactive. The steam distillate, on the other hand, was still lævo-rotatory, reducing, and gave the Voges and Proskauer reaction. An osazone was prepared from this distillate and gave a definite melting point of 243° C., corresponding to the phenylosazone of diacetyl, which is always obtained from acetylmethylcarbinol in this manner. In a similar way the action of

B. mesentericus vulgatus and *Tyrothrix tenuis* on mannitol and glucose respectively was also examined and similar results obtained.

The Action of Tyrothrix tenuis *on Glycerol.*

This offered a case of much greater interest, as the formation of acetylmethylcarbinol would involve a carbon synthesis, and does not take place from this substance when *B. lactis aërogenes* is used under anaërobic conditions.

Péré's directions were again carefully followed, 5 grm. of glycerol were made up to 100° C. with broth, and, after sterilisation and subsequent inoculation, were incubated at 37° C. for 30 days. An investigation of the culture medium, as described above, again showed the presence of acetylmethylcarbinol, which passed over into the distillate, and was characterised by the preparation and analysis of the osazone, as well as by the Voges and Proskauer reaction. The lead, calcium, and barium salts of the oxidation products analysed by Péré, and believed by him to be the salts of glyceric acid, were in all probability the salts of lactic acid. As he was only able to analyse extremely small quantities (0·032 grm. of a lead salt containing 50 per cent. Pb) of non-crystalline substances, the experimental error was probably too great for him to distinguish between these two acids. There is therefore no evidence to show that glyceraldehyde is produced in the above fermentations, and the theory that sugars undergoing bacterial fermentation are first broken down to trioses derives no support from this investigation.

The Action of B. lactis aërogenes (*Escherich*) *on Glycerol under Anaërobic Conditions.*

The experiments previously described were all carried out under aërobic conditions, and, as an example of anaërobic decomposition, the action of *B. lactis aërogenes* on glycerol has been studied. This organism under these conditions forms acetylmethylcarbinol from all the hexoses, but produces none from glycerol, no reducing substance at all being found among the products of the reaction.

The method of investigation was substantially that used by Harden (8), the gases, however, being collected and measured in the apparatus devised by Harden, Thompson, and Young (9). The organism was grown in an atmosphere of nitrogen, the medium consisting of 1 per cent. Witte's peptone solution containing 10 per cent. of glycerol, sufficient chalk being added to neutralise the acids formed during fermentation. In the glycerol experiments quoted, the times of growth were respectively five weeks,

two weeks, and four weeks. A different strain of the organism was used in Experiment 1 from that used in Experiments 2 and 3.

The glycerol added, and the amount unfermented, were estimated by means of the method of Zeisel and Fanto (10), as described below.

Estimation of the Residual Glycerol.—100 c.c. of the liquid in which the organism had grown were evaporated at a low temperature under reduced pressure, in order to ensure the removal of all alcohol. The residue was then taken up in a small quantity of water, the volume made up to 100 c.c. with water, and 5 c.c. of this solution taken for the estimation.

Search for Acetylmethylcarbinol.—A portion of the culture medium was distilled and the distillate tested for reducing power with Fehling's solution. In every case the distillate was found to be non-reducing and the absence of acetylmethylcarbinol was further confirmed by trying the Voges and Proskauer reaction, which was invariably negative. It was also found impossible to prepare any osazone.

Detection of 2.3-*Butylene Glycol.*—This substance was detected and estimated as previously described, the estimation being made on 300 c.c. of the culture medium in which the organism had been grown. In every experiment this glycol was found to be present, the other products being ethyl alcohol, formic, lactic, and succinic acids, carbon dioxide, and hydrogen.

The diacetylphenylosazone prepared from the oxidation product of the butylene glycol was analysed and gave the following results:—

0·1372 grm. substance gave 0·3656 grm. CO_2 and 0·0876 grm. H_2O. C = 72·6 per cent.; H = 7 per cent.

0·0950 grm. substance gave 17·5 c.c. N at 21° C and 765·5 mm. N = 21·12 per cent.

$C_{16}H_{18}N_4$ requires C = 72·2 per cent., H = 6·8 per cent., N = 21·05 per cent.

The lactic acid produced was also characterised by the preparation and analysis of the zinc salt and by Fletcher and Hopkins' reaction (11).

0·1500 grm. of the zinc salt dried at 105° C. gave 0·0499 grm. ZnO. ZnO = 33·28 per cent.

$(C_3H_5O_3)_2Zn$ requires ZnO = 33·4 per cent.

The analysis of the calcium salt prepared from the succinic acid formed gave the following results:—

0·0550 grm. substance gave 0·0202 grm. CaO. CaO = 36·7 per cent.

$(C_4H_4O_4)Ca$ requires CaO = 35·9 per cent. The pure acid was also isolated in this case and gave M.P. 183–4° C.

The percentage of these substances on the weight of glycerol fermented is shown in the following table, Columns 1, 2, and 3. Columns 4 and 5

show for comparative purposes the result of the action of *B. lactis aërogenes* on glucose and mannitol respectively.

Table I.

	Glycerol.			Glucose.	Mannitol.
	1.	2.	3.	4.	5.
Alcohol	35·2	37·7	37·3	17·1	32·5
Acetic acid.....	6·1	5·0	7·3	5·1	2·5
Lactic acid.....	13·0	12·7	11·13	5·5	8·6
Succinic acid	4·05	1·6	4·03	2·4	3·2
2.3-Butylene glycol ...	9·9	Not estimated		(27·2)	(12·0)
Formic acid	6·38	4·9	7·5	1·0	1·5
Carbon dioxide	22·4	28·37	31·8	38·0	35·5
CO_2, c.c. per grm.....	110·6	144·0	160·7	198·3	180·3
H_2, c.c. per grm.	79·8	139·0	156·7	82·4	138·3
Ratio H_2/CO_2.....	0·72	0·97	0·97	0·42	0·77

The figures in brackets are estimated from other experiments.

Table II shows the number of carbon atoms per molecule of glycerol decomposed, represented by each product.

Table II.

	Glycerol.			Glucose.	Mannitol.
	1.	2.	3.	4.	5.
Alcohol	1·42	1·43	1·49	1·34	2·57
Acetic acid.....	0·18	0·15	0·22	0·31	0·15
Lactic acid.....	0·40	0·39	0·34	0·33	0·52
Succinic acid	0·12	0·05	0·12	0·15	0·20
2.3-Butylene glycol ...	0·39	Not estimated		(2·17)	(0·97)
Formic acid and CO_2	0·58	0·69	0·82	1·64	1·53
Total	3·09	2·71	2·99	5·94	5·94
Hydrogen, atoms per molecule	0·65	1·13	1·28	1·33	2·26

Columns 4 and 5 are again comparative ones of *B. lactis aërogenes* on glucose and mannitol.

It is interesting to find that alcohol accounts for 35·2 per cent. of the glycerol used, as against 17·1 per cent. in the case of glucose. Harden suggested (8) that the source of the alcohol might be the presence in the molecule undergoing decomposition of the terminal group $CH_2(OH)\cdot CH(OH)$–. This was confirmed in the case of glucose and mannitol, this latter substance yielding twice the amount of alcohol produced under similar conditions from

glucose. It would be interesting to find whether the same relationship holds in the case of glycerol and glyceraldehyde. Formic acid (or its decomposition products, CO_2 and H_2) and alcohol, which might be formed according to the equation

$$C_3H_8O_3 = C_2H_6O + CH_2O_2,$$

make up 64—77 per cent. of the glycerol fermented.

Summary.

1. The volatile reducing substance obtained by Péré in the aërobic fermentation of mannitol by *B. subtilis* and *B. mesentericus vulgatus*, and of glucose and glycerol by *Tyrothrix tenuis*, is acetylmethylcarbinol, which is readily volatile in steam, gives the Voges and Proskauer reaction, and forms the phenylosazone of diacetyl.

2. The action of *B. lactis aërogenes* on glycerol, under anaërobic conditions, does not give rise to any reducing substance.

The products of this decomposition have been quantitatively estimated and are as follows:—Ethyl alcohol, formic, acetic, lactic and succinic acids, carbon dioxide, hydrogen and 2.3-butylene glycol.

[*Note added February* 29, 1912.—Since writing the foregoing paper our attention has been called to a paper by Fernbach* in which he shows that *T. tenuis* acts both on glucose and glycerol with the production of non-volatile dihydroxyacetone. Volatile reducing substances were also formed which he regards as a mixture of methylglyoxal and formaldehyde. Since neither of these substances is optically active they cannot be identical with the lævo-rotatory substance obtained by Péré and ourselves, so that Fernbach's observations in no way disprove our conclusion that the optically active, volatile substance produced is acetylmethylcarbinol.]

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* 'Compt. Rend.,' 1910, vol. 151, p. 1004.