XL.—*Decomposition of Mannitol and Dextrose by the Bacillus ethaceticus.*

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IN previous communications, the fermentative changes brought about by this specific microbe, in suitable solutions of mannitol, glycerol, and calcium glycerate, have been described by one of us. It has been shown that in all these cases, almost the exclusive products, excepting the gaseous ones, are ethyl alcohol and acetic acid, together with varying proportions of formic acid and traces of succinic acid. The object of the present investigation was, firstly, to determine the nature of the gaseous products, and, secondly, to endeavour to ascertain the quantitative relationship existing between the various products of these fermentations, in the same manner as has been recently done by one of us for the fermentations induced by the *Pneumococcus* (Friedländer) in mannitol and dextrose.

The methods adopted were substantially the same as those described in the latter communication, a given quantity of the 3 per cent. solutions of mannitol and dextrose, respectively, being fermented in bottles provided with delivery-tubes, so as to admit of the collection of the evolved gases over mercury. The volume of gas evolved in each case was measured, and representative samples were submitted to analysis, whilst in the liquid left after fermentation was complete, the alcohol and volatile acids were determined, according to the methods already fully described.

Fermentation of Mannitol.

400 c.c. of a 3 per cent. solution of mannitol, to which the necessary nutritive materials had been added, were fermented with a pure culture of the *B. ethaceticus* at a temperature of 36°.

In the following table the volumes of gas collected during the course of the fermentation are recorded :—

No. of days after inoculation.	Volume of gas.		Total volume of gas collected.
4	Tube	(1) 28·4 c.c.	28·4 c.c.
4	„	(2) 37·0 „	65·4 „
4	* „	(3) 13·2 „	78·6 „
5	„	(4) 100·0 „	178·6 „
5	„	(5) 17·8 „	196·4 „
6	„	(6) 90·2 „	286·6 „

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No. of days after inoculation.	Volume of gas.	Total volume of gas collected.
8	Tube (7) 131.2 c.c.	417.8 c.c.
10	" (8) 116.0 "	533.8 "
12	" (9) 89.0 "	622.8 "
15	*,, (10) 126.7 "	749.5 "
19	" (11) 80.0 "	829.5 "
26	" (12) 120.7 "	950.2 "
28	" (13) 25.2 "	975.4 "
30	" (14) 24.4 "	999.8 "
31	" (14a) 21.4 "	1021.2 "
33	*,, (14b) 19.2 "	1040.4 "
36	" (15) 29.8 "	1070.2 "
43	" (16) 53.0 "	1123.2 "
68	*,, (17) 126.0 "	1249.2 "
75	" (18) 7.8 "	1257.0 "

Thus, the total volume of gas collected amounted to 1257 c.c.

The progress of the fermentation is most clearly indicated by means of the following graphic representation, in which the abscissæ denote lapse of time in days since inoculation, and the ordinates, the volume of gas measured in cubic centimetres:—

Mr. McCowan, B.Sc., Lecturer on Mathematics and Physics in University College, Dundee, has again most kindly come to our assistance in the investigation of the mathematical properties of this curve, of which he finds the equation to be

$$v = \frac{11.1t^2}{1 + 0.049t + 0.00843t^2},$$

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in which v = volume of gas in cubic centimetres, and t = time in days from the date of inoculation.

The manner in which the curve fulfils the conditions of this equation may be gathered from the following comparisons of the results of calculation and observation :—

Day.	Volume of gas (calculated from equation).	Volume of gas (observed).
5	190 c.c.	78·6 c.c.
10	475 „	480·0 „
20	829 „	829·5 „
30	994 „	990·0 „
40	1080 „	1090·0 „
50	1131 „	1170·0 „
60	1162 „	1210·0 „

Thus, just as in the case of the curve representing the evolution of gas in the fermentation of mannitol by the *Pneumococcus*, the results of observations are below those of calculation at the beginning, and above them at the close, of the fermentation, which is doubtless, to a great extent, due to the absorption of carbonic anhydride by the liquid at the commencement, and by the continued decomposition of bicarbonate of lime at the termination of the process.

Samples of the gas thus given off were submitted to analysis, and in the preceding table (pp. 432—433) the tubes selected for examination are marked with a *.

Mannitol Fermentation.

	Composition of gases evolved on				Mean (excluding that of the 4th day).
	4th day.	12th—15th day.	31st—33rd day.	43rd—68th day.	
CO ₂	47·36	58·03	60·50	57·26	58·59
O	0·30	0·06	0·06	0·32	0·15
H	48·76	41·89	39·36	42·20	41·15
N	3·58	0·02	0·08	0·22	0·11
	100·00	100·00	100·00	100·00	100·00

The gaseous products are thus exclusively carbonic anhydride and hydrogen, just as has already been shown to be the case in the fermentation of mannitol with the *Pneumococcus* (Friedländer); moreover, as in that case also, the gases are given off in about the proportion of 6 volumes or molecules of carbonic anhydride to 4 volumes

or molecules of hydrogen. In taking the average above we have, of course, excluded the gas collected on the fourth day, when air was still present, and when absorption of carbonic anhydride by the liquid was obviously still going on.

In the liquid, after the fermentation was completed, the alcohol and volatile acids were determined as previously described; the alcohol being first oxidised with potassium dichromate and sulphuric acid, and the acetic acid so formed being then converted into barium salt, whilst the volatile acids were also converted into barium salts, weighed, and analysed.

Thus the acid obtained by oxidation of the alcohol yielded 3·3835 grams of barium salt, yielding 90·92 per cent. BaSO_4 .

This corresponds to 1·221 gram of ethyl alcohol.

The volatile acids gave 1·601 gram of barium salt, yielding 97·46 per cent. BaSO_4 , corresponding to

0·8652	gram of barium formate	=	0·3505	gram of formic acid.
0·7358	„ „ acetate	=	0·3463	„ „ acetic „
<hr/>				
1·6010				

As regards the gaseous products, assuming the whole of the 1257 c.c. of gas collected to have the composition indicated by the average figures given above (58·59 per cent. CO_2 , 41·15 per cent. H_2), there would be 736·6 c.c. of carbonic anhydride or 1·452 gram, and 517·9 c.c. of hydrogen or 0·046 gram. Of the above carbonic anhydride, it can be calculated that 0·2946 gram is due to the decomposition of calcium carbonate by the acetic and formic acids liberated, whilst the remaining 1·1574 gram is due to the actual fermentation of the mannitol. As recently pointed out by one of us (this vol., p. 270), the whole of the hydrogen with its molecular equivalent of carbonic anhydride may in these fermentations be referred to a preliminary liberation of formic acid which has subsequently undergone decomposition, $\text{CH}_2\text{O}_2 = \text{CO}_2 + \text{H}_2$. Such preliminary liberation of formic acid is attested by the fact that a very variable quantity of formic acid is found amongst the products of fermentation; and in the present instance the amount of formic acid which had thus escaped decomposition was relatively very large. It is obvious, therefore, that in computing the fermentation products, the decomposed formic acid (represented by the hydrogen + molecular equivalent of carbonic anhydride) should be added to the formic acid actually found. Adopting this plan, the fermentation products may be thus tabulated:—

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C_2H_6O	1.2210 gram.
$C_2H_4O_2$	0.3463 „
CH_2O_2 { 0.3505 gram found	} 1.4085 „
H_2 (found) .. 0.046 gram	
CO_2 (0.046 $\times$ 22) 1.012 „	(calculated)
CO_2 1.1574 gram	— 1.0120 = 0.1454 „
(Due to fermenta- tion.)	(Due to decomposed formic acid.)

In endeavouring to interpret these results, it must be borne in mind that the figures for carbonic anhydride must almost certainly be somewhat low, owing to a portion of the latter remaining dissolved in the liquid and thus escaping determination. The proportions in which the other products are formed are in very fair harmony with the following equation:—

this involves the proportions—

$C_2H_4O_2$.	$5C_2H_6O$.	$5CH_2O_2$.	CO_2 .
60	230	230	44
1	: 3.8	: 3.8	: 0.7

Experimental proportions—

1	: 3.5	: 4.1	: 0.4
Acetic acid.	Alcohol.	Formic acid.	Carbonic anhydride.

These proportions correspond, moreover, most strikingly with those obtained in one out of the two similar experiments made by one of us with the *Pneumococcus* of Friedländer on mannitol (Trans., 1891, 59, 253), thus:—

1	: 3.7	: 4.1	: 0.7
Acetic acid.	Alcohol.	Formic acid.	Carbonic anhydride.

On the other hand, a second and more incomplete fermentation of mannitol with the *Pneumococcus* of Friedländer (*loc. cit.*) gave discordant results, thus:—

1	: 2.2	: 2.5	: 0
Acetic acid.	Alcohol.	Formic acid.	Carbonic anhydride.

Fermentation of Dextrose.

Two experiments made in this case were of a precisely similar nature.

First Experiment.—430 c.c. of a 3 per cent. solution of dextrose

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containing also the necessary nutritive materials were fermented with a pure culture of *B. ethaceticus*, the fermentation proceeding side by side in the same incubator at 36° with that of the mannitol described above. The evolution of gas was as follows:—

No. of days after inoculation.	Volume of gas.	Total volume of gas collected.
4	Tube (1) 44.0 c.c.	44.0 c.c.
4	„ (2) 18.8 „	62.8 „
5	* „ (3) 90.0 „	152.8 „
6	„ (4) 60.0 „	212.8 „
10	* „ (5) 130.0 „	342.8 „
12	„ (6) 75.0 „	417.8 „
15	* „ (7) 102.1 „	519.9 „
19	„ (8) 66.0 „	585.9 „
26	* „ (9) 118.5 „	704.4 „
28	„ (10) 24.6 „	729.0 „
30	„ (11) 29.1 „	758.1 „
31	„ (12) 30.0 „	788.1 „
33	* „ (13) 24.0 „	812.1 „
36	„ (14) 46.0 „	858.1 „
43	„ (15) 99.0 „	957.1 „
51	„ (16) 76.0 „	1033.1 „
68	* „ (17) 106.4 „	1139.5 „
75	„ (18) 6.0 „	1145.5 „

Thus the total volume of gas collected amounted to 1145.5 c.c. as compared with 1257.0 c.c. in the fermentation of the mannitol.

We may again indicate the progress of the fermentation by means of a graphic representation in which the abscissæ show the lapse of time in days from the inauguration of the fermentation, and the ordinates the volume of gas evolved, measured in cubic centimetres:—

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On comparing this curve No. 1 with that for the mannitol fermentation (p. 433), it will be seen that it is much flatter than the latter, although it is essentially of the same character as is shown by the equation which has been kindly calculated by our friend Mr. McCowan. Thus the convex portion of the curve is defined by the following equation

$$v = \frac{11t^2}{1 + 0.197t + 0.0068t^2},$$

in which v = volume of gas in cubic centimetres, and t = time in days from the date of inoculation.

The coincidence between calculation and observation, excepting at the extremities of the curve, where the same departures as before are noticeable, is again very close, thus:—

Day.	Volume of gas (calculated from equation).	Volume of gas (observed).
5	128 c.c.	62.8 c.c.
10	301 "	330.0 "
20	574 "	585.0 "
30	759 "	770.0 "
40	890 "	900.0 "
50	988 "	1020.0 "
60	1061 "	1100.0 "

In the second fermentation of dextrose (see p. 437), the form of the curve is much more like that of the mannitol curve.

Specimens of the gas evolved at different stages of the fermentation were submitted to analysis, the particular tubes being marked * in the table on p. 437. Thus:—

Dextrose Fermentation. No. 1.

	Composition of gases evolved on						Mean excluding that of 4th—5th day.
	4th—5th day.	6th—10th day.	12th—15th day.	19th—26th day.	31st—33rd day.	51st—68th day.	
CO ₂	47.45	60.32	69.86	62.30	61.43	55.91	61.96
O.....	0.10	0.11	0.10	0.08	0.13	0.55	0.19
H.....	35.21	39.04	30.06	37.63	38.43	42.28	37.49
N.....	17.24	0.53	0.00	0.00	0.01	1.26	0.36
	100.00	100.00	100.02	100.01	100.00	100.00	100.00

From the average composition of the evolved gas, it appears that the proportion of carbonic anhydride to hydrogen is distinctly greater than in the case of the gas given off in the fermentation of mannitol.

Assuming that the whole of the 1145·5 c.c. of gas collected had the average composition of 61·96 per cent. carbonic anhydride and 37·49 per cent. of hydrogen, there would be 710 c.c. or 1·400 gram of carbonic anhydride and 430 c.c. or 0·039 gram of hydrogen.

From the liquid, after the close of the fermentation, there were obtained, after oxidation of the alcohol, 2·115 grams of barium salt, yielding 90·97 per cent. of barium sulphate and corresponding to 0·7632 gram of ethyl alcohol; and, from the volatile acids, 2·026 grams of barium salt, yielding on analysis 94·09 and 94·19 per cent. of barium sulphate, corresponding to a mixture of 0·7191 gram of acetic acid with 0·2018 gram of formic acid.

Before summarising the results of this experiment, we would refer to the

Second Experiment, which was made under similar conditions but at quite a different time. The solution fermented contained:—dextrose, 12 grams; peptone, 0·4 gram; salt solution, 40 c.c.; calcium carbonate, 4 grams; the whole being diluted to 400 c.c. The temperature during the greater part of the fermentation was 37—38°, whilst after it was practically complete, the temperature accidentally rose above 40°; the evolution of gas took place as indicated below:—

No. of days after inoculation.	Total volume of gas collected in c.c. (corrected to 0° and 760 mm.).	
4	45·4	Temperature 37—38°.
5	187·0	
6	305·0	
7	473·6	
9	665·1	
13	755·9	
20	840·0	
34	957·0	
39	979·4	
45	1002·6	
47	1006·3	
66	1034·0	Total.

Samples of the evolved gas were, from time to time, submitted to analysis, with the following results:—

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	I. 5th day.	II. 8th day.	III. 12th day.	IV. 33rd day.	V. 39th day.	Mean.
CO ₂	67·79	59·70	57·30	65·38	66·20	63·27
O 0·10	0·10	0·08	0·14	0·40	0·02	0·15
H 31·42	31·42	40·12	42·61	33·52	33·76	36·29
N 0·69	0·69	0·10	0·00	0·70	0·02	0·30
	100·00	100·00	100·05	100·00	100·00	100·01

The average composition of the gas is thus almost identical with that evolved in the first experiment. Assuming that the whole of the 1034 c.c. of gas collected contained the average 36·29 per cent. hydrogen, and that the remainder was wholly composed of carbonic anhydride (thus neglecting the insignificant traces of oxygen and nitrogen found), there would be 658·8 c.c. carbonic anhydride or 1·2986 gram, and 375·2 c.c. hydrogen or 0·0336 gram.

From the liquid after the close of the fermentation, there was obtained, by oxidation of the alcohol, 1·3665 gram of barium salt, yielding 90·28 per cent. and 90·14 per cent. BaSO₄, corresponding to 0·493 gram of ethyl alcohol; and from the volatile acids, 1·758 gram of barium salt, yielding 93·94 per cent. and 93·92 per cent. BaSO₄, which corresponds to a mixture of 0·159 gram of formic acid with 0·641 gram of acetic acid. Making, again, the same calculations as described in the case of the mannitol fermentation (see p. 435), the results of these two dextrose fermentations may be summarised as follows:—

Fermentation of Dextrose.

I. (430 c.c. of 3 per cent. Dextrose Solution.)

C ₂ H ₆ O	0·7632 gram.
C ₂ H ₄ O ₂	0·7191 „
CH ₂ O ₂ {	0·2018 gram (found).....
H ₂ (found) 0·039 gram.....	0·039 gram.....
CO ₂ (0·039 × 22) 0·858 gram {	0·897 gram (calculated)
CO ₂ 1·0398 gram —	0·8580 gram = 0·1818 gram.
(Due to fermenta- tion.)	(Due to decomposed formic acid.)

II. (400 c.c. of 3 per cent. Dextrose Solution.)

C ₂ H ₆ O	0·8375 „
C ₂ H ₄ O ₂	0·6410 „
CH ₂ O ₂ {	} 0·9328 gram.
0·1600 gram (found)	
H ₂ (found) 0·0336 gram	
CO ₂ (0·0336 × 22) 0·7392 gram {	0·7728 gram (calculated)

$$\text{CO}_2 \dots 0.9876 \text{ gram} - 0.7392 \text{ gram} = 0.2484 \text{ gram.}$$

(Due to fermenta- (Due to decomposed
tion.) formic acid.)

We have evidence that what is recorded above as carbonic anhydride (over and above that due to decomposed formic acid) is derived from the decomposition of the calcium carbonate by an unidentified, fixed acid, insoluble in ether. For in the second of these two experiments we have determined the total calcium carbonate which passed into solution during fermentation, and found it to be 1.368 gram, corresponding to 0.6019 gram carbonic anhydride, which represents the carbonic anhydride liberated by the action of all the acids formed on the calcium carbonate. But the carbonic anhydride, due to the action of the formic and acetic acids can be calculated to be 0.3110 gram, leaving 0.2909 gram of carbonic anhydride, due to the decomposition of the calcium carbonate by the unidentified acid. This point is particularly interesting, as, if the above 0.6019 gram carbonic anhydride, due to decomposition of the calcium carbonate, be deducted from the total carbonic anhydride found by gas analysis, namely, 1.2986 gram, the remainder, 0.6967 gram of carbonic anhydride, is almost exactly what is required (viz., 0.7392 gram CO_2) to produce formic acid with the hydrogen found. The fermentation thus consists of the decomposition of the dextrose with formation of ethyl alcohol, acetic acid, formic acid (the greater part of which decomposes into carbonic anhydride and hydrogen), an unidentified fixed acid, insoluble in ether, together with traces of succinic acid, the presence of which we have found in subsequent experiments described below. The several products appear in the following molecular proportions:—

Fermentation of Dextrose.

	No. 1.		No. 2.		Molecules (mean).
	Weights Molecular weights	Molecules.	Weights Mol-ecular weights	Molecules.	
$\text{C}_2\text{H}_6\text{O}$	0.0166	2.2	0.0182	2.8	2.5
$\text{C}_2\text{H}_4\text{O}_2$	0.0120	1.6	0.0107	1.6	1.6
CH_2O_2	0.0239	3.1	0.0203	3.1	3.1
CO_2 equivalent of unidentified acid }	(0.0076)*	1	0.0066	1	1

* As the CO_2 equivalent of the unidentified acid was not determined in this fermentation, it has been here calculated, assuming this acid to have been formed in the same proportion to the acetic and formic acids as in Fermentation No. 2.

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The coincidence between the results obtained in these two fermentations, although carried on with an interval of six months between them, is very striking.

We have also carried out a number of fermentations of dextrose with *B. ethaceticus* in open flasks (plugged with cotton wool), in which, of course, the gaseous products had to be neglected. The results of these fermentations may be summarised as follows:—

No. 1.—60 grams of dextrose in 2000 c.c. of liquid were fermented. The fermentation lasted about five weeks.

By oxidation of the alcoholic portion, there were obtained—

Barium salts, 16·062 grams (yielding 91·32 per cent. BaSO_4), corresponding to *ethyl alcohol* 5·794 grams.

The volatile acids gave 11·9135 grams of barium salt (yielding 90·39 per cent. BaSO_4), corresponding to *acetic acid* 5·606 grams.

	Weights Molecular weights	=	Molecules.
$\text{C}_2\text{H}_6\text{O}$	0·126		1·4
$\text{C}_2\text{H}_4\text{O}_2$	0·093		1·0

No. 2.—60 grams of dextrose in 2000 c.c. of liquid were submitted to fermentation. The fermentation lasted only about three weeks. By oxidation of the alcoholic portion there were obtained—

Barium salts 19·5315 grams (yielding 91·41 per cent. BaSO_4), corresponding to *ethyl alcohol* 7·047 grams.

The volatile acids gave—

Fraction I. 7·244 grams of barium salt (yielding 96·39 per cent. BaSO_4).

Fraction II. 3·228 grams of barium salt (yielding 95·27 per cent. BaSO_4).

Which corresponds to—

3·989 grams of barium formate = 1·617 gram *formic acid*.

6·483 grams of barium acetate = 3·050 grams *acetic acid*.

	Weights Molecular weights	=	Molecules.
$\text{C}_2\text{H}_4\text{O}$	0·153		3·0
$\text{C}_2\text{H}_4\text{O}_2$	0·051		1·0
CH_2O_2	0·035		0·7

This is the only one of these four fermentations conducted in open flasks in which we have observed the production of any considerable proportion of formic acid; in the remaining three, formic acid was

practically absent. The exceptional production of formic acid in open fermentations of mannitol has previously been observed by one of us, and is in this case not improbably connected with the unusually short duration of the fermentation.

No. 3.—30 grams of dextrose in 1000 c.c. of liquid were employed for fermentation; the fermentation lasted about 7 weeks.

By oxidation of the alcoholic portion there were obtained:—

Barium salts 8.145 grams (yielding 91.24 per cent. BaSO_4), corresponding to *ethyl alcohol* 2.939 grams.

The volatile acids gave—

3.488 grams of barium salts (yielding 89.70 per cent. BaSO_4), corresponding to 1.641 *gram of acetic acid*.

	Weights Molecular weights	=	Molecules.
$\text{C}_2\text{H}_6\text{O}$	0.064		2.4
$\text{C}_2\text{H}_4\text{O}_2$	0.027		1.0

No. 4.—30 grams of dextrose in 1000 c.c. of liquid were employed for fermentation. The fermentation lasted about six weeks.

By oxidation of the alcoholic portion there were obtained—

Barium salts 7.159 grams (yielding 90.59 per cent. BaSO_4), corresponding to *ethyl alcohol* 2.582 *grams*.

The volatile acids gave—

5.876 grams of barium salts (yielding 91.29 per cent. BaSO_4), corresponding to 2.765 *grams of acetic acid*.

	Weights Molecular weights	=	Molecules.
$\text{C}_2\text{H}_6\text{O}$	0.056		1.2
$\text{C}_2\text{H}_4\text{O}_2$	0.046		1.0

The results of our investigation may be summarised as follows:—

1. The principal products of the fermentation of mannitol and dextrose by *B. ethaceticus* are ethyl alcohol, acetic acid, carbonic anhydride, and hydrogen.

2. When the fermentation is conducted in a closed space, as when the gases evolved are collected over mercury, formic acid is also an invariable product, whilst in fermentations conducted in an open space (*e.g.*, flasks plugged with cotton wool), formic acid is an exceptional product. This may be accounted for by supposing that some of the carbonic anhydride and hydrogen evolved are in the first instance liberated in the shape of formic acid, and that this undergoes less complete decomposition when it is constrained to remain in contact

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with its decomposition products—carbonic anhydride and hydrogen—as is the case when the fermentation is conducted in a closed space.

3. The decomposition of the mannitol may be thus represented —

Even in the fermentations conducted in a closed space, the greater part of the formic acid in question is decomposed, whilst in open fermentations the whole generally undergoes complete resolution into carbonic anhydride and hydrogen, formic acid in any quantity being only exceptionally found amongst the products.

4. In the case of the fermentation of dextrose we have shown that there is formed, in addition to the above products, a fixed acid insoluble in ether, which we have not yet identified, but the carbonic anhydride equivalent of which we have determined.

The products of the dextrose fermentation are liberated in the following molecular proportions:—

C_2H_6O	2.5 molecules.
$C_2H_4O_2$	1.6 „
CH_2O_2	3.1 „
CO_2 equivalent of unidentified acid	1.0 „

Thus in the fermentation of dextrose the proportion of acetic acid to alcohol and formic acid is greater than in the case of the mannitol. In the case of the mannitol fermentation, it is almost certain, also, that there is a fixed acid insoluble in ether formed in addition to the products indicated, and the molecule of carbonic anhydride given in the equation above doubtless represents its equivalent, and has been obtained through the decomposition of calcium carbonate by this unidentified acid.

5. The fermentations in a closed space are always found to be very markedly less complete than those conducted under circumstances permitting of the free diffusion of the gaseous products.

6. The close qualitative and quantitative resemblance which we have established as existing between the decompositions brought about by *B. ethaceticus* and the *Pneumococcus* point to this ethacetic fermentation being a very general one which not improbably can be brought about by a number of different microorganisms.