67. Origin and Relationship of Acetylmethylcarbinol to 2:3-Butylene Glycol in Bacterial Fermentations*

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No general agreement exists as to the mechanism of the biological origin of acetylmethylcarbinol and 2:3-butylene glycol, although these products are formed by the dissimilation of various carbohydrates and polyhydric alcohols by many species of bacteria.

The purpose of the present communication is to contribute to our knowledge of the mechanism of the formation of acetylmethylcarbinol and 2:3-butylene glycol by *Aerobacillus polymyxa* (Prazmowski) Donker, an organism which produces considerable quantities of these products. Studies were made using as cultural media both glucose-peptone and glucose-peptone plus several probable intermediary compounds.

Desmots [1904] found acetylmethylcarbinol in cultures of *Bacillus mesentericus*. Organisms which produce acetylmethylcarbinol generally form 2:3-butylene glycol, the reduction product of the carbinol. Harden & Walpole [1906] isolated and described both 2:3-butylene glycol and acetylmethylcarbinol from the fermentation of glucose by *Aerobacter aerogenes*. Harden & Norris [1912] found that *Aerobacter aerogenes* and *Aerobacter cloacae*, when grown in a peptone solution containing any one of a series of sugars and polyhydric alcohols, produce both the carbinol and the glycol. Lemoigne [1919; 1923] found the carbinol to be formed by species of *Proteus* and by *Bacillus anthracis*. Neuberg & Reinfürth [1923], Kluyver, Donker & Visser't Hooft [1925] and Elion [1926] found it in yeast cultures. Lemoigne [1912], Lafon [1932], and Horowitz-Wlassowa & Rodionowa [1933] showed that the carbinol was present in cultures of *Bacillus subtilis*; van Niel, Kluyver & Derx [1929] detected it in cultures of *Streptococcus cremoris*, and first suggested the importance to flavour and aroma of the presence of acetylmethylcarbinol in butter cultures. Michaelian, Farmer & Hammer [1933] have conducted extensive investigations to show the role of diacetyl in butter aroma. Visser't Hooft & de Leeuw [1935] found the carbinol in bread which had a desirable aroma. These citations are but a few of the recorded examples.

Investigators are not agreed on the origin of acetylmethylcarbinol. Considerable evidence has been presented to support the view that acetaldehyde is an intermediary in the fermentation of glucose to acetylmethylcarbinol. Neuberg & Hirsch [1921], Neuberg & Ohle [1922], Neuberg & Reinfürth [1923], and Neuberg & Simon [1925] presented data showing that the carbinol is formed by the condensation of one molecule of added aldehyde with one molecule of biologically formed aldehyde. Neuberg attributes this condensation of aldehyde to the enzyme carboligase. In the normal yeast fermentation of glucose, no acetylmethylcarbinol or 2:3-butylene glycol is formed. Kluyver & Donker [1925] believe that acetaldehyde is a normal intermediate in this fermentation but that it is removed so rapidly that no condensation occurs. These investigators added other H acceptors, such as O₂, methylene blue and S, to compete with acetaldehyde, and in each case acetylmethylcarbinol and 2:3-butylene glycol were formed.

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Furthermore there is no general agreement as to whether at least one molecule of acetaldehyde must be nascent to form acetylmethylcarbinol. Elion [1926], starting with a 10% yeast suspension in 3% acetaldehyde, obtained a fair quantity of acetylmethylcarbinol in 3 hr. Barritt [1937] inoculated *Aerobacter aerogenes* and *Escherichia coli* into peptone water containing varying concentrations of acetaldehyde. Growth occurred in some of the media but acetylmethylcarbinol was not detected in any.

Formation of acetylmethylcarbinol from pyruvic acid by *Acetobacter* has been shown by Hermann & Neuschul [1932], and by *Clostridium acetobutylicum* by Johnson, Peterson & Fred [1933]. Pyruvic acid may be decarboxylated to form acetaldehyde, and the latter condense with pyruvic acid or with another molecule of itself to acetylmethylcarbinol.

Silverman & Werkman [1941], however, reported that a cell-free enzyme preparation from *Aerobacter aerogenes* converts pyruvic acid into acetylmethylcarbinol and CO_2 without detectable intermediate formation of acetaldehyde. It should be emphasized that the mechanisms of dissimilation with proliferating cells and with enzyme preparations may differ and that different species of bacteria may use different mechanisms of formation.

Other investigators differ as to the origin of acetylmethylcarbinol. Horowitz-Wlassowa & Rodionowa [1933] believe that with *Bacillus subtilis*, 2:3-butylene glycol results directly from splitting of the sugar molecule and that acetylmethylcarbinol is a product of biological oxidation of 2:3-butylene glycol. Lafon [1932] could detect neither pyruvic acid nor acetaldehyde in glucose cultures of *Bacillus subtilis* which produce acetylmethylcarbinol. He believed that the carbinol may originate from lactic acid or that, perhaps, there is a splitting of the hexose molecule to give the C_4 compound directly.

Several investigators have presented evidence that acetylmethylcarbinol originates from 2:3-butylene glycol by biological oxidation in the absence of a fermentable carbohydrate. Walpole [1910] was able to demonstrate the formation of the carbinol by cultivating *Aerobacter aerogenes* in a medium composed only of 2:3-butylene glycol, peptone and water. Lemoigne [1923] noted an increase of acetylmethylcarbinol, and a decrease of 2:3-butylene glycol in a *Proteus* culture after it was held for 18 days. Donker [1926] obtained the carbinol by growing *Aerobacillus polymyxa* aerobically in a medium composed only of yeast-water and 2:3-butylene glycol. Werkman [1930] inoculated a medium of 2:3-butylene glycol, $(\text{NH}_4)_2\text{SO}_4$, and K_2HPO_4 with several species of *Aerobacter*. Acetylmethylcarbinol was detected in cultures of all of the organisms on the second day. Barritt [1937] found that many bacteria can oxidize 2:3-butylene glycol to acetylmethylcarbinol and suggested that in the bacterial fermentation of glucose the glycol may precede the formation of the carbinol.

On the other hand, there is evidence that in the presence of fermentable sugar the reverse process takes place, namely, the reduction of acetylmethylcarbinol to 2:3-butylene glycol. Neuberg & Kobel [1925] added acetylmethylcarbinol to a fermenting mixture of sugar and yeast; the carbinol had disappeared by the third day and 2:3-butylene glycol was isolated from the fermented mixture. Nagelschmidt [1927] found acetylmethylcarbinol to be an intermediary product of the reduction of diacetyl to 2:3-butylene glycol by yeast. Visser't Hooft & de Leeuw [1935] found that under certain conditions the acetylmethylcarbinol in bread is reduced to 2:3-butylene glycol. According to Hammer, Stahly, Werkman & Michaelian [1935], under certain conditions the citric acid-fermenting streptococci of butter cultures reduce acetylmethylcarbinol to 2:3-butylene glycol.

Methods and materials

Acetylmethylcarbinol was determined by the method outlined by Stahly & Werkman [1936]. 2:3-Butylene glycol was determined according to Brockmann & Werkman [1933] and a correction made for the presence of any acetylmethylcarbinol [Stahly & Werkman, 1936]. Ethyl alcohol was determined according to Stahly, Osburn, & Werkman [1934]

with correction for acetylmethylcarbinol. The volatile acids were determined by the method of Osburn, Wood & Werkman [1933].

Culture. In all the experiments described in this paper, *Aerobacillus polymyxa* (Prazmowski) Donker, [Donker, 1926] was used (culture no. 842 of the American Type Culture Collection).

EXPERIMENTAL

Effect of addition of acetaldehyde and acetate on yields of acetylmethylcarbinol and 2:3-butylene glycol

Freshly distilled aldehyde was added to a vigorously fermenting medium of 2% glucose, 1% CaCO_3 , 0.5% peptone and 0.2% K_2HPO_4 . The acetaldehyde, 0.708 g./300 ml. of medium, was added 40 hr. after inoculation. The controls contained basal medium without acetaldehyde. During the fermentation some of the media were aerated; others received a constant stream of O_2 -free N_2 . Some of the acetaldehyde may have escaped during the fermentation; none remained at the close.

The yields of 2:3-butylene glycol, acetylmethylcarbinol and ethyl alcohol (Table 1) were definitely greater in those cultures which received acetaldehyde. Aeration resulted in greater yields of acetylmethylcarbinol, both in the control and in the dissimilations receiving acetaldehyde, than occurred in strictly anaerobic experiments. Evidently added acetaldehyde is utilized by *Aerobacillus polymyxa* under the conditions of the experiment. Ethyl alcohol results, apparently, from the reduction of acetaldehyde.

Table 1. *Quantities of acetylmethylcarbinol, 2:3-butylene glycol and ethyl alcohol produced by Aerobacillus polymyxa in a glucose medium with and without added acetaldehyde*

Figures represent g. per 100 ml. of fermented liquor.

Condition	Medium	Glucose fermented	A.M.C.	2:3-B.G.	Total A.M.C. + 2:3-B.G.	Ethyl alcohol
Anaerobic	Control	1.96	0.017	0.483	0.501	0.342
"	Control + acetaldehyde	1.99	0.050	0.580	0.631	0.472
"	"	1.98	0.034	0.529	0.563	0.491
Aerobic	Control	1.99	0.062	0.482	0.545	0.225
"	Control + acetaldehyde	1.94	0.069	0.522	0.591	0.383
"	"	1.85	0.065	0.577	0.642	0.385

A.M.C. = acetylmethylcarbinol.

2:3-B.G. = 2:3-butylene glycol.

Additional evidence for the intermediary role of acetaldehyde was secured by the use of CaSO_3 as a fixing agent. A medium composed of 1% CaCO_3 , 0.2% K_2HPO_4 , 0.5% peptone, 2% glucose and 1% CaSO_3 was inoculated with *Aerobacillus polymyxa* and incubated at 30°. The fermented liquor was then distilled to one-half volume into a dilute ice-cold solution of dimedon. Crystallization of the derivative occurred slowly after several hours in the refrigerator. Melting point determinations indicated that the substance fixed was acetaldehyde.

An experiment was designed to determine whether the addition of acetate to a normal glucose fermentation by *Aerobacillus polymyxa* results in an increase of acetylmethylcarbinol and 2:3-butylene glycol. Both anaerobiosis and aeration were employed. The control medium was 2% glucose, 1% CaCO_3 , 0.5% peptone and 0.2% K_2HPO_4 . Calcium acetate, 2 g./300 ml.; was added to the basal medium. Glucose was determined immediately after inoculation; and glucose, acetylmethylcarbinol and 2:3-butylene glycol after fermentation was complete. Table 2 gives typical experimental results.

It is apparent that added acetate results in a marked increase in the yield of 2:3-butylene glycol and acetylmethylcarbinol in the fermentation of glucose by *Aerobacillus*

Table 2. *Quantities of acetylmethylcarbinol and 2:3-butylene glycol produced by Aerobacillus polymyxa in a glucose medium with and without added acetate*

Figures represent g. per 100 ml. of fermented liquor.

Condition	Medium	Glucose fermented	A.M.C.	2:3-B.G.	A.M.C. + 2:3-B.G.
Anaerobic	Control	2.03	0.034	0.388	0.422
"	Control + acetate	1.97	0.028	0.554	0.582
Aerobic	Control	1.97	0.036	0.466	0.502
"	Control + acetate	1.98	0.071	0.626	0.697
"	"	1.99	0.073	0.630	0.703

polymyxa both aerobically and anaerobically. In every trial the total yield of acetylmethylcarbinol plus 2:3-butylene glycol was greater when calcium acetate was added. Aeration resulted in increased yields of acetylmethylcarbinol, both in the controls and in the medium receiving acetate. The results indicate that acetic acid plays a part in the biological formation of 2:3-butylene glycol and acetylmethylcarbinol. The intermediate mechanism is not evident; the acetic acid may first be reduced to acetaldehyde, and the latter may then condense with itself or pyruvic acid to acetylmethylcarbinol, which would be reduced to 2:3-butylene glycol.

The relationship of acetylmethylcarbinol to 2:3-butylene glycol in the glucose fermentation by Aerobacillus polymyxa

Experiments were designed to determine the fate of both acetylmethylcarbinol and 2:3-butylene glycol when they are added separately to glucose dissimilations by *Aerobacillus polymyxa*. Preliminary studies showed that vigorous oxygenation of a glucose culture of *Aerobacillus polymyxa* resulted in rapid fermentation, and that if acetylmethylcarbinol were added during the course of the fermentation, it was reduced largely to the corresponding glycol. In an attempt to secure a better understanding of the changes involved, it was decided to make serial determinations of glucose, acetylmethylcarbinol, 2:3-butylene glycol and E_h values during the course of typical fermentations. The medium was composed of 2% glucose, 1% CaCO_3 , 0.3% peptone and 0.2% K_2HPO_4 .

Three 2000 ml. Erlenmeyer flasks, each containing 1200 ml. of medium were fitted with five-hole rubber stoppers which held platinum electrode, KCl-agar bridge, gas outlet tube, gas inlet tube leading into an aerator device, and sampling tube. The apparatus was arranged to determine the redox potentials of the biological system according to the method of Werkman, Johnson & Coile [1933]. O_2 was continuously bubbled through the medium immediately after inoculation with *Aerobacillus polymyxa* until fermentation was completed. The cultures were incubated at 30°. Flask no. 1 served as the control; to flask no. 2 was added a definite quantity of sterile acetylmethylcarbinol 46 hr. after inoculation; and to flask no. 3 was added sterile 2:3-butylene glycol, 46 hr. after inoculation. The data are presented in Tables 3, 4 and 5.

The correlation of acetylmethylcarbinol and 2:3-butylene glycol with unfermented glucose and with E_h values is apparent from Fig. 1 *a* and *b*. The data are from Tables 3, 4 and 5.

Fig. 1 *b* represents the data obtained with flask 2. The results are typical of the two other fermentations. The data of this experiment show that in all three fermentations there was a rapid drop in redox potentials concomitant with a rapid decrease in the concentration of glucose. While the E_h value remained low, very little acetylmethylcarbinol accumulated, but there was a rapid rise in the quantity of 2:3-butylene glycol. In flask 1 (control) a marked increase in acetylmethylcarbinol and decrease in the rate of production of 2:3-butylene glycol occurred at about the 60th hr. The redox potential had become fairly high at that time, and the glucose was almost completely fermented.

The glycol content reached a maximum at the 70th hr. and then began to decrease. This decrease was almost exactly accounted for by the increase in acetylmethylcarbinol.

Table 3. *Quantities of acetylmethylcarbinol, 2:3-butylene glycol and glucose; and E_h values at intervals during the course of the fermentation of glucose by Aerobacillus polymyxa*

Flask 1—control.					
Hours after inoculation	Unfermented glucose g./100 ml.	E_h , volts	Molarity A.M.C.	Molarity 2:3-B.G.	Molarity A.M.C. + 2:3-B.G.
0	1.99	0.113	0.0	0.0	0.0
24	1.19	-0.041	0.0005	0.0200	0.0205
32	—	-0.037	—	—	—
46	0.44	-0.047	0.0020	0.0461	0.0481
70	0.0	—	0.0102	0.0539	0.0641
80	0.0	+0.027	—	—	—
94	0.0	+0.200	0.0209	0.0429	0.0638
127	0.0	+0.177	—	—	—
142	0.0	+0.177	0.0244	0.0377	0.0621

Table 4. *Quantities of acetylmethylcarbinol, 2:3-butylene glycol and glucose; and E_h values at intervals during the course of the fermentation of glucose by Aerobacillus polymyxa*

Flask 2—A.M.C. added.					
Hours after inoculation	Unfermented glucose g./100 ml.	E_h , volts	Molarity A.M.C.	Molarity 2:3-B.G.	Molarity A.M.C. + 2:3-B.G.
0	1.96	+0.247	0	0	0
8	—	+0.262	—	—	—
24	1.26	-0.233	0.0003	0.0198	0.0201
32	—	-0.266	—	—	—
46	0.30	-0.196	0.0020-0.0239*	0.0470	0.0490-0.0709
70	0.04	+0.093	0.0219	0.0646	0.0854
127	0.0	+0.307	—	—	—
142	0.0	+0.322	0.0325	0.0521	0.0846

* Sterile A.M.C. was added.

Table 5. *Quantities of acetylmethylcarbinol, 2:3-butylene glycol and glucose; and E_h values at intervals during the course of the fermentation of glucose by Aerobacillus polymyxa*

Flask 3—2:3-B.G. added.					
Hours after inoculation	Unfermented glucose g./100 ml.	E_h , volts	Molarity A.M.C.	Molarity 2:3-B.G.	Molarity A.M.C. + 2:3-B.G.
0	1.87	+0.267	0	0	0
8	—	+0.327	—	—	—
24	1.26	-0.223	0.0008	0.0194	0.0202
32	—	-0.199	—	—	—
46	0.44	-0.173	0.0026	0.0465-0.0939*	0.0491-0.0965
70	0.0	—	0.0122	0.0947	0.1069
80	0.0	+0.089	—	—	—
94	0.0	+0.274	0.0263	0.0817	0.1080
127	0.0	+0.377	—	—	—
142	0.0	+0.382	0.0276	0.0793	0.1069

* Sterile 2:3-B.G. added.

The graphs for the fermentation in flask 2 show a definite decrease in the quantity of acetylmethylcarbinol after the carbinol was added and while the E_h value was still fairly low. This decrease was accounted for by the increase in glycol content in excess of that in flask 1 to which no carbinol had been added. To flask 3 was added 2:3-butylene glycol at the 46th hr. after inoculation. There was a slight rise after this in the glycol content but not as much as in flask 1. The rise in acetylmethylcarbinol occurred earlier and to a greater extent in flask 3 than in flask 1.

It is interesting to note that (1) passage of a stream of O_2 through these fermenting liquors did not prevent a rapid drop in E_h ; (2) acetylmethylcarbinol added to the fermenting medium was quickly reduced to the corresponding glycol while the E_h remained low; (3) after most of the glucose had been fermented and the E_h had risen appreciably,

Fig. 1 a.

Fig. 1 a. Correlation of acetylmethylcarbinol and 2:3-butyleneglycol with unfermented glucose and with E_h . Data for flasks nos. 1, 2 and 3.

Fig. 1 b.

Fig. 1 b. Correlation of acetylmethylcarbinol and 2:3-butyleneglycol with unfermented glucose and with E_h . Data for flask 2.

much 2:3-butyleneglycol was oxidized to the carbinol; and (4) after all the glucose had been fermented the total content of acetylmethylcarbinol plus 2:3-butyleneglycol did not change significantly.

It is evident that a low redox potential favours the reduction of carbinol to glycol and a high potential favours the oxidation of the glycol to carbinol. It is near or at the time of the final disappearance of fermentable carbohydrate that conditions become suitable for the accumulation of acetylmethylcarbinol in the *Aerobacillus polymyxa* fermentation.

SUMMARY AND CONCLUSIONS

1. In fermentations by *Aerobacillus polymyxa*, conducted either aerobically or anaerobically, the addition of acetaldehyde to a glucose-containing medium results in increased yields of ethyl alcohol, acetylmethylcarbinol and 2:3-butyleneglycol.

2. Acetaldehyde is fixed by the addition of $CaSO_3$ to a normal fermentation. This evidence supports the probable intermediary role of the aldehyde.

3. Likewise, the addition of acetate results in increased yields of acetylmethylcarbinol and 2:3-butyleneglycol.

4. A marked drop in redox potential and a rapid fermentation of the glucose follow inoculation of a vigorously oxygenated glucose medium with *Aerobacillus polymyxa*. The potential rises only after most of the glucose has been fermented.

5. Large quantities of 2:3-butyleneglycol accumulate while the redox potential remains low; part of the glycol is oxidized to acetylmethylcarbinol after the E_h attains a fairly high level.

6. Little acetylmethylcarbinol accumulates during the early stages of the fermentation; added carbinol is reduced to glycol but only when the potential is low.

7. It is suggested that acetylmethylcarbinol and 2:3-butylene glycol comprise a reversible oxidation-reduction system. A low redox potential favours the accumulation of the glycol; a high potential favours carbinol formation.

REFERENCES

- Barritt, M. M. [1937]. *J. Path. Bact.* **44**, 679.
 Brockmann, M. & Werkman, C. H. [1933]. *Industr. Engng Chem.* (Anal. ed.), **5**, 206.
 Desmots, H. [1904]. *C.R. Acad. Sci., Paris*, **138**, 581.
 Donker, H. J. L. [1926]. *Bijdrage tot de kennis der boterzuurbutylalcoholen acetongistingen*. Delft, Holland: W. D. Meinema.
 Elion, L. [1926]. *Biochem. Z.* **171**, 40.
 Hammer, B. W., Stahly, G. L., Werkman, C. H. & Michaelian, M. B. [1935]. *Res. Bull. Iowa Agric. exp. Sta.* no. 191.
 Harden, A. & Norris, D. [1912]. *Proc. roy. Soc. B*, **84**, 492.
 Harden, A. & Walpole, G. S. [1906]. *Proc. roy. Soc. B*, **77**, 399.
 Hermann, S. & Neuschul, P. [1932]. *Biochem. Z.* **246**, 446.
 Horowitz-Wlassowa, L. M. & Rodionowa, E. A. [1933]. *Zbl. Bakt.* **II**, **87**, 333.
 Johnson, M. J., Peterson, W. H. & Fred, E. B. [1933]. *J. biol. Chem.* **101**, 145.
 Kluyver, A. J. & Donker, H. J. L. [1925]. *Akad. Wet. Amst. Proc.* **28**, 314.
 Kluyver, A. J., Donker, H. J. L. & Visser't Hooft, F. [1925]. *Biochem. Z.* **161**, 361.
 Lafon, M. [1932]. *Bull. Soc. Chim. biol., Paris*, **14**, 263.
 Lemoigne, M. [1912]. *C.R. Acad. Sci., Paris*, **155**, 792.
 — [1919]. *C.R. Soc. Biol., Paris*, **82**, 984.
 — [1923]. *C.R. Soc. Biol., Paris*, **88**, 467.
 Michaelian, M. B., Farmer, R. S. & Hammer, B. W. [1933]. *Res. Bull. Iowa Agric. exp. Sta.* no. 155.
 Nagelschmidt, G. [1927]. *Biochem. Z.* **186**, 317.
 Neuberg, C. & Hirsch, J. [1921]. *Biochem. Z.* **115**, 282.
 Neuberg, C. & Kobel, M. [1925]. *Biochem. Z.* **160**, 250.
 Neuberg, C. & Ohle, H. [1922]. *Biochem. Z.* **128**, 610.
 Neuberg, C. & Reinfürth, E. [1923]. *Biochem. Z.* **143**, 553.
 Neuberg, C. & Simon, E. [1925]. *Biochem. Z.* **156**, 374.
 Osburn, O. L., Wood, H. G. & Werkman, C. H. [1933]. *Industr. Engng Chem.* (Anal. ed.), **5**, 247.
 Silverman, M. & Werkman, C. H. [1941]. *J. Bact.* **41**, 22.
 Stahly, G. L., Osburn, O. L. & Werkman, C. H. [1934]. *Analyst*, **59**, 319.
 Stahly, G. L. & Werkman, C. H. [1936]. *Iowa St. Coll. J. Sci.* **10**, 205.
 van Niel, C. B., Kluyver, A. J. & Derx, H. B. [1929]. *Biochem. Z.* **210**, 234.
 Visser't Hooft, F. & de Leeuw, F. J. G. [1935]. *Cereal Chem.* **12**, 213.
 Walpole, G. S. [1910]. *Proc. roy. Soc. B*, **83**, 272.
 Werkman, C. H. [1930]. *J. Bact.* **20**, 121.
 Werkman, C. H., Johnson, C. A. & Coile, H. D. [1933]. *Iowa St. Coll. J. Sci.* **7**, 163.