THE PRODUCTION OF ACETYLMETHYLCARBINOL BY AEROBACTER FROM A VARIETY OF CARBON SOURCES IN A SYNTHETIC MEDIUM¹

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Although considerable work has been done on the formation of acetylmethylcarbinol by *Aerobacter*, except for the work of Koser (1918), O'Meara (1931), and Reynolds and Werkman (1937a,b), those studies employing cultures of growing cells were done in complex, chemically undefined media which tend to obscure the underlying chemical reaction. In the present study, intact, washed *Aerobacter* cells were used as the inoculum to determine acetylmethylcarbinol formation from a variety of carbohydrates, sugar alcohols, and related compounds, each of which was the sole carbon source in a synthetic, chemically defined medium.

MATERIALS AND METHODS

A basal salt solution of the following composition was prepared: $\text{NaNH}_4\text{HPO}_4 \cdot 4\text{H}_2\text{O}$ (Eimer and Amend reagent), 1.5 g; KH_2PO_4 (Mallinckrodt reagent), 1.0 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (Eimer and Amend reagent), 0.4 g; distilled water q. s., 1,000 ml. This solution was sterilized in the autoclave at 15 pounds pressure for 15 minutes and had a pH of 6.7 after sterilization. The various carbon sources were made up as 10 per cent solutions in distilled water and sterilized in 10 ml quantities by filtration through a Seitz filter. Five ml of such a sterile solution were added aseptically to 95 ml of the sterile basal salt solution to yield 100 ml of a medium containing 0.5 per cent of the substance under study. This medium then was dispensed aseptically in 5.0 ml quantities in sterile 15 by 125 mm screw-capped culture tubes.

Six strains of *Aerobacter* were employed. Cul-

tures no. 12, 14, and 100 were identified as *Aerobacter aerogenes*, no. 100 being ATCC 8308. Cultures no. 11, 13, and 15 were identified as *Aerobacter cloacae*. These cultures were carried on nutrient agar slants at a pH of 6.8. After incubation for 24 hours at 30 C, growth from such a slant was washed twice at 4,000 rpm for 15 minutes in 5.0 ml physiological saline. The cells were suspended then in 5.0 ml physiological saline, of which 0.1 ml was used to inoculate each tube of medium containing the carbon source under study. These cultures were incubated at 30 C for 48 hours.

The tests were run as follows: Since there was no source of guanidine groups in the medium and since Harden and Norris (1911) had shown the guanidine group to be necessary to secure a positive test, dihydrostreptomycin sulfate (Schenley) was used as a source of guanidine groups. Approximately 5 mg of dihydrostreptomycin were added to a 16 by 150 mm test tube, following the method of O'Meara (1931) who used creatine. Then, following the method of Barritt (1936), one ml of culture was added to the above tube, followed by 0.6 ml of 5.0 per cent α -naphthol in absolute ethanol and 0.2 ml of 40 per cent KOH. The tubes were shaken after the addition of the reagents and were read after five minutes. Positive tests, indicating production of acetylmethylcarbinol, were pink, gradually turning deep red, while negative tests were colorless, gradually turning yellow.

Controls were run on all phases of the tests. Sterility was checked by incubating each tube of medium before use and also by inoculating a small portion of each medium onto blood agar slants. A test was run on an uninoculated tube of each medium to determine that there was no substance present which would give a false positive test. To determine that the inoculum alone would not give a positive test, a heavy suspension of bacteria from a freshly prepared nutrient

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TABLE 1

The production of acetylmethylcarbinol by Aerobacter species from a single carbon source in a synthetic medium

CARBON SOURCE	CULTURE NO.					
	11	12	13	14	15	100
Glycerol.....	—	+	—	+	—	+
D-(—)-Erythrose.....	0	0	0	0	0	0
L-Arabinose.....	+	+	+	+	+	+
D-Ribose.....	0	+	+	+	+	+
L-Xylose.....	+	+	+	+	+	+
Glucose.....	+	+	+	+	+	+
D-Glucosamine·HCl..	+	+	+	+	+	+
D-Galactose.....	+	+	—	+	—	+
D-Fructose.....	+	+	+	+	+	+
D-Mannose.....	+	+	+	+	+	+
Mannitol.....	+	+	+	+	+	+
Adonitol.....	0	0	0	0	0	0
Inositol.....	0	—	—	—	—	—
D-Sorbitol.....	+	+	+	+	+	+
L-Rhamnose.....	+	+	+	+	+	+
Sucrose.....	+	+	+	+	+	+
Lactose.....	0	+	0	+	0	+
Cellobiose.....	+	+	+	+	+	+
Maltose.....	+	+	+	+	+	+
Glycogen.....	0	0	0	0	0	0
Melezitose.....	0	0	0	0	0	0
D-Glucosaminoglycose.....	0	0	0	0	0	0
D-Glucoheptulose.....	0	0	0	0	0	0
Sodium acetate.....	0	0	0	0	0	0
Acetaldehyde.....	0	0	0	0	0	0
Pyruvic acid.....	0	0	0	0	0	0
Ascorbic acid.....	0	0	0	0	0	0
DL-Alanine (1 per cent).....	—	—	—	—	—	—

* Weak.

+ = positive test for acetylmethylcarbinol.

— = negative test for acetylmethylcarbinol.

0 = no growth, or very scanty growth.

agar slant was made up in distilled water; when tested for acetylmethylcarbinol by the method described above, this preparation gave negative results. A positive control was run with each carbon source tested to determine that the reagents were satisfactory.

Sterile, exogenous acetylmethylcarbinol was added, before inoculation, to fresh tubes of those media in which growth had occurred but which gave a negative test for acetylmethylcarbinol (table 1). This was done to determine if acetylmethylcarbinol had been produced but rendered

undetectable due to other fermentation products. These cultures were retested after incubation with acetylmethylcarbinol present and gave positive results in all cases.

According to Harden (1906), acetylmethylcarbinol is oxidized to diacetyl under the conditions of our experiment and is the compound actually tested for, rather than acetylmethylcarbinol. The sensitivity of our test was one part of diacetyl in 100,000 and was obtained by testing one ml amounts of 10-fold dilutions of diacetyl in place of the one ml of culture. In all other respects, the test was identical with the procedures indicated above.

RESULTS AND DISCUSSION

The results indicate that under the conditions of the experiment strains of growing *Aerobacter* can form acetylmethylcarbinol from a variety of carbohydrates, sugar alcohols, and related compounds, each of which is the sole carbon source in a synthetic, chemically defined medium. In general, there is a close correlation between our results in synthetic media and those previously reported in complex media, as will be shown below.

Harden and Norris (1912), using complex media, claimed that *A. aerogenes* did not form acetylmethylcarbinol from glycerol. These authors used chalk to neutralize the acids formed in the medium and thus may have kept the pH too high for acetylmethylcarbinol formation. They also used very long incubation periods and possibly allowed any acetylmethylcarbinol which may have been formed to be reduced to 2:3-butylene glycol, which they did discover in the medium. Koser (1918) found, however, that in a synthetic medium containing glycerol, uric acid, and mineral salts, 50 strains of *A. aerogenes* gave an equal number of positive Voges-Proskauer tests. Our results, using a medium even more simple than that used by Koser, are in agreement with his results.

Our results with arabinose, glucose, galactose, fructose, mannose, and mannitol in synthetic media are in agreement with the results found by Harden and Norris (1911-1912) in complex media. Our results with xylose in synthetic media verify similar results in synthetic media found by Reynolds and Werkman (1937b).

The positive results with 5 out of 6 cultures in the utilization of glucosamine are in agreement

with the results found both by Koser and Saunders (1933) and by Wedum and Walker (1935), who reported utilization of this compound in complex media but did not determine the end products. In a report made while the present work was in progress, Matsushima and Shimazu (1952) stated that *A. cloacae* could utilize glucosamine as a sole carbon source in a synthetic medium, with production of 2:3-butylene glycol, after 10 days' incubation at 37 C. A shorter incubation period at a lower temperature probably would have revealed the production of acetylmethylcarbinol had the tests been made.

Adonitol failed to support growth under the conditions of our experiment although Harden and Norris (1911-1912) claimed that fermentation of this compound in complex media by *Aerobacter* did result in the production of acetylmethylcarbinol.

Using manometric techniques, Volk and Pennington (1951, 1952) and Magasanik (1951) suggested a pathway for inositol metabolism differing from the glucose pathway. Our results support their conclusions since no acetylmethylcarbinol was found although inositol was utilized by our cultures.

Our results showing nonutilization of the heptoses tested are in agreement with previous findings regarding heptose utilization reported by Koser and Saunders (1933) and by Sternfeld and Saunders (1937), who used complex media.

Acetic acid was tested to determine if its utilization would result in the production of acetylmethylcarbinol since this acid has been suggested as a precursor of acetylmethylcarbinol. It was tested in the form of its sodium salt, and our results showing its nonutilization in a synthetic medium are in agreement with the results reported by Reynolds *et al.* (1937), who found that sodium acetate was not fermented by *Aerobacter indologenes* even in a complex medium. They found that glucose was a necessary constituent of the medium, which also contained peptone, in order that sodium acetate be utilized.

Our results showing nonutilization of acetaldehyde in a synthetic medium are in agreement with Barritt (1937), who found that a concentration of added preformed acetaldehyde, such as was used in our experiment, inhibited growth of *A. aerogenes* even in complex media.

Dozois *et al.* (1936) showed that *A. aerogenes* could utilize ascorbic acid in complex media but did not determine the end products. *Aerobacter*

evidently cannot utilize this compound under the conditions of our experiment.

Barritt (1937) claimed that in a complex medium, pyruvate was converted to acetylmethylcarbinol by growing *A. aerogenes*, but this compound failed to support growth under the conditions of our experiment.

The negative results obtained with DL-alanine show that this compound is not converted to acetylmethylcarbinol by *Aerobacter* and indicate that alanine, generally considered to be glucogenic in higher forms of life, is not so with *Aerobacter* under the conditions of the experiment.

In two papers submitted while the present work was in progress, Halliday (1952) and Szafir and Bennett (1953) both reported a color reaction of streptomycin and dihydrostreptomycin, when in aqueous alkaline solution and mixed with diacetyl and α -naphthol. These findings are in agreement with our use of dihydrostreptomycin as a source of guanidine groups in our test.

Thus, with the exception of our results with glycerol, our positive tests for acetylmethylcarbinol in synthetic media agree with results previously reported by other workers when they tested these same compounds in complex media.

In addition, ribose, glucosamine, sorbitol, rhamnose, sucrose, lactose, cellobiose, and maltose have never before been reported to give a positive test for acetylmethylcarbinol when utilized by *Aerobacter*.

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SUMMARY

A study has been made of the production, by *Aerobacter aerogenes* and *Aerobacter cloacae*, of acetylmethylcarbinol (when tested for by the method of Barritt) from a variety of compounds, each of which was the sole carbon source in a synthetic, chemically defined medium.

The compounds which gave a positive test for acetylmethylcarbinol, when utilized by *Aerobacter* species in synthetic media, agreed with the results previously reported using complex media.

In addition, eight compounds never before reported to give a positive test for acetylmethylcarbinol were found to give such a positive test when utilized by *Aerobacter* species.

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