EFFECT OF ALDEHYDES AND FATTY ACIDS AS ADDED HYDROGEN ACCEPTORS ON THE FERMENTATION OF GLUCOSE BY AEROBACTER INDOLOGENES

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The use of suitable hydrogen acceptors added to a normal fermentation may permit of certain conclusions as to the normal path of fermentation, especially if the acceptor can be traced and the effects of its reduction on the normal dissimilation observed.

Harden and Norris (1902) reported the formation of butylene glycol by *Bacillus lactis-aerogenes* (*Aerobacter aerogenes*) from acetaldehyde in a 1 per cent peptone medium in the absence of glucose. However, most of the acetaldehyde was recovered as acetic and succinic acids and ethyl alcohol.

Neuberg and Kobel (1925) noted that 1 molecule of acetaldehyde added to yeast preparations in the presence of glucose or pyruvic acid condensed with a molecule of acetaldehyde biologically formed to yield acetylmethylcarbinol.

Kluyver and Donker (1925) were able to cause yeast and lactic acid bacteria to form acetylmethylcarbinol from glucose when methylene blue or sulphur was added as a hydrogen acceptor.

Whetham (1927) believed the presence of acetylmethylcarbinol was a reliable indication of the intermediary occurrence of acetaldehyde.

Hammer (1936) found that the addition of homologs of acetaldehyde to fermenting milk cultures of *Streptococcus liquefaciens* resulted in an increase in acetylmethylcarbinol but that homologs of acetylmethylcarbinol were not formed.

Reynolds, Jacobsson and Werkman (1937) noted that acetic acid, when added to a glucose fermentation by *Aerobacter indologenes*, was reduced and the yield of hydrogen suppressed. The reduced acetate was quantitatively accounted for as 2,3-butylene glycol.

The purpose of the present study was to determine the effect of homologs of acetaldehyde and acetic acid on the fermentation of glucose by *Aerobacter*. The results should be of considerable importance to an understanding of the formation of acetylmethylcarbinol and 2,3-butylene glycol. If a homolog of acetic acid, e.g., propionic, were reduced and the product condensed to the carbinol and further reduced to glycol, it is reasonable to expect that 4-carbon-, 5-carbon- and possibly 6-carbon-glycols would be formed. Since, on oxidation the glycols yield the corresponding acids from which they are derived, it is possible to determine whether such a condensation occurs. Likewise for higher aldehydes a similar procedure can be applied. Use of the higher acids and aldehydes offers an advantage over acetic acid and acetaldehyde in that their reduction products can be traced.

In the results to be reported, acetaldehyde, propionaldehyde, butyraldehyde and acetic, propionic, butyric and fumaric acids were added to glucose fermentations and their effect on dissimilation studied.

METHODS

Fermentations were conducted at 30°C. in 500 ml. Erlenmeyer flasks fitted with dropping funnels for the addition of the acids and aldehydes. The medium was 2.0 per cent glucose, 0.3 per cent $(NH_4)_2SO_4$, 1.0 per cent K_2HPO_4 and 0.05 per cent peptone. Ten per cent (by volume) of tap water was added for the salts present. The medium was autoclaved and after inoculation flushed with oxygen-free nitrogen before connecting to the bottles for the absorption of gases.

Freshly distilled aldehydes were added in approximately 0.6 M concentration. The added aldehyde was determined by titration of bound bisulphite with iodine according to Donnally (1933).

Acids used were weighed, neutralized to bromothymol blue, made up to the desired concentration and determined by steam-distilling an aliquot part and titrating.

The fumaric acid was weighed, neutralized, sterilized by Seitz filtration and determined gravimetrically as the silver salt.

The inoculum was prepared by growing *Aerobacter indologenes* on nutrient agar and using an aqueous suspension for inoculation. Cultures were examined before and after fermentation by the gram stain.

Carbon dioxide and hydrogen were collected in bottles containing 1 M alkali, the carbon dioxide being absorbed in the alkali and the hydrogen determined by volume displacement. Residual carbon dioxide was determined gravimetrically by acidifying the medium to congo red and refluxing an aliquot part in a stream of CO_2-free air.

After removal of carbon dioxide the sample was distilled to half volume, the distillate neutralized and again distilled to half volume to recover alcohols. The two residues were combined, acidified to congo-red paper and steam-distilled to collect volatile acids. The volatile acids in the distillate were determined by the partition method according to Osburn, Wood and Werkman (1933). Formic acid was determined by the method of Auerback and Zeglin (1922).

Alcohols were determined by oxidizing an aliquot part of the neutral volatile distillate with H_2SO_4—$K_2Cr_2O_7$ mixture in a closed flask in a boiling water bath, steam-distilling and determining the resulting acids on the steam distillate by the partition method.

Acetylmethylcarbinol was determined according to Stahly and Werkman (1935). 2,3-Butylene glycol was separated from the medium by steam distillation from a neutral medium saturated with $MgSO_4 \cdot 7H_2O$. The glycol was determined on the distillate by oxidizing with periodic acid and directly distilling the resulting acetaldehyde into sodium bisulphite solution and titrating with iodine.

Lactic acid was determined on the residue of the butylene glycol distillation by the method of Friedemann and Graesser (1933).

Residual aldehydes were determined by distilling an aliquot part of the fermentation directly into bisulphite and titrating with iodine.

To determine whether any of the added aldehydes or acids had condensed to the corresponding glycols, the glycol distillate was oxidized, as in the procedure for determining alcohol, and the resulting steam distillate partitioned to detect the presence of acids other than acetic. By this procedure butylene glycol is oxidized quantitatively to acetic acid, each mole yielding two of the acid.

RESULTS

It was first desirable to determine the fate of acetaldehyde added to fermenting cultures of *Aerobacter indologenes* under acid and alkaline conditions (table 1). It has previously been shown (Mickelson and Werkman, 1938; also see table 1) that the pH of the medium has a very pronounced effect on the formation of butylene glycol. The addition of acetaldehyde to an alkaline fermentation results in its equimolar conversion into acetic acid and ethyl alcohol. The work of Reynolds and Werkman (1936) suggested that acetic acid may be an intermediate in the formation of 2,3-butylene glycol with acetaldehyde and acetylmethylcarbinol acting as intermediates in the transformation. If *Aerobacter indologenes* contains carboligase which will couple acetaldehyde under conditions when added acetic acid is not attacked (alkaline reaction), it should be possible to demonstrate whether acetic acid is necessary in the formation of butylene glycol. The results obtained do not bear out this hypothesis (table 1).

When the aldehyde is added to a fermentation conducted at a pH level favorable to the formation of butylene glycol there is a substantial increase in acetylmethylcarbinol (table 1) and likewise ethyl alcohol and acetic acid. It appears that the added aldehyde has been in part condensed to the carbinol. Since acetaldehyde can serve as a precursor of acetic acid,

TABLE 1

Effect of adding aldehydes to glucose fermentations of Aerobacter indologenes

	ALDEHYDE FERMENTED	CO_2	H_2	FORMIC ACID	ACETIC ACID	LACTIC ACID	ACETYLMETHYL-CARBINOL	2,3-BUTYLENE GLYCOL	ETHYL ALCOHOL		SUCCINIC ACID	BUTYRIC ACID	CARBON RECOVERY	O/R INDEX
													per cent	
Control	0	164.00	41.50	16.40	0.66	4.30	1.15	64.50	58.20				95.5	0.98
Acid acetaldehyde	43.20	174.00	8.46	0.76	9.13	2.64	17.66	66.40	63.80				96.7	1.06
Alkaline acetaldehyde	43.00	10.10	2.73	167.20	109.80	8.80	0	1.36	109.30				94.3	1.03
										PROPYL ALCOHOL				
Propionaldehyde	24.20	173.50*	41.20*	8.65	1.00	3.70	0.32	68.00	32.40	27.20	5.15		93.5	1.05
										BUTYL ALCOHOL				
Butyraldehyde	22.50	175.00	41.20	13.00		4.32	0.65	65.60	50.40	14.40	3.52	5.65	97.5	1.01

Products in mM per 100 mM fermented glucose.

* Calculated, sample lost.

TABLE 2

Effect of adding acids to glucose fermentations of Aerobacter indologenes

	ACID FERMENTED	CO_2	H_2	FORMIC ACID	ACETIC ACID	LACTIC ACID	ACETYLMETHYL-CARBINOL	2, 3-BUTYLENE GLYCOL	ETHYL ALCOHOL	PROPYL ALCOHOL	SUCCINIC ACID	ACETIC ACID ADDED	CARBON RECOVERY	O/R INDEX
Control	0	164.00	41.50	16.40	0.66	4.30	1.15	64.50	58.20				95.5	0.98
Acetic acid	36.00	174.00	0	9.00	50.00	3.00	2.20	88.00	55.00			86.00	98.6	0.95
Propionic acid	27.50	163.00	14.90	17.70	8.60	2.70	0.64	75.00	21.60	27.50			93.0	1.02
Fumaric acid	18.50	165.20	35.60	17.50	0.67	2.92	0.32	66.00	48.50		24.10		97.0	1.01

Products in mM per 100 mM fermented glucose.

ethyl alcohol, acetylmethylcarbinol or butylene glycol, there is no assurance that condensation has occurred because the added aldehyde has not been tagged and its fate determined.

Higher aldehydes and acids offer the advantage that it is possible to trace them. If synthetic benzaldehyde is coupled with biologically formed acetaldehyde to give phenylacetylcarbinol as Neuberg and Ohle (1922) found to be the case with yeast, it may be expected that *Aerobacter* will couple added propionaldehyde or butyraldehyde with acetaldehyde to give the corresponding carbinols. The data show that such is not the case. Instead, the added aldehydes are oxidized and reduced to the corresponding acids and alcohols with no increase in acetylmethylcarbinol. These results are to be compared with those of Hammer (1935) who obtained increases in acetylmethylcarbinol by the addition of homologs of acetaldehyde to cultures of *Streptococcus liquefaciens*. Likewise, those substances which do not cause increased yields of the carbinol or glycol with *Aerobacter* do not decrease the yield of hydrogen as do acetaldehyde and propionic and acetic acids.

Most of the work on the origin of acetylmethylcarbinol in bacterial and yeast fermentations has emphasized that one mole of the acetaldehyde must be nascent, i.e., of biological origin. Tomiyasu (1936) claims to have obtained a synthesis of acetylmethylcarbinol with a yeast preparation from synthetic acetaldehyde and states that nascent aldehyde from sugar or pyruvic acid is unnecessary. Recent work by Discherl (1937) points to the necessity of having nascent aldehyde formed before acetylmethylcarbinol synthesis can take place from synthetic acetaldehyde. He attributes the results of Tomiyasu to the lack of a yeast preparation free of endogenous fermentation.

When acetaldehyde was added to cell suspensions of the organism in buffer at a pH favorable to the formation of acetylmethylcarbinol and 2,3-butylene glycol from glucose no condensation of aldehyde occurred. Apparently the formation of acetylmethylcarbinol, if it occurs by a condensation of acetaldehyde, is an energy-consuming reaction and, since the side reaction providing the energy is not taking place, no formation of acetylmethylcarbinol is possible. It is also possible that the acetaldehyde must couple with some other intermediate in order that the formation of the carbinol may occur.

If the cells cannot synthesize acetylmethylcarbinol from synthetic acetaldehyde it is not readily conceivable that a similar compound of biological origin would be more readily converted. Perhaps, as suggested by Discherl (1931), the acetaldehyde couples first with pyruvic acid and yields acetylmethylcarbinol with a loss of carbon dioxide. At any rate it may not be necessary that the compound of biological origin be nascent acetaldehyde.

Propionaldehyde and butyraldehyde do not cause increases in acetylmethylcarbinol or butylene glycol. In either case there is a small amount of succinic acid formed, accompanied by a decrease in ethyl alcohol (table 1). Propionaldehyde caused a greater reduction in the yield of ethyl alcohol and was itself entirely converted to propyl alcohol. With butyraldehyde some butyric acid was formed.

Unless *Aerobacter indologenes* is unable to synthesize higher glycols, it is not unreasonable to suppose that acetaldehyde is reduced to the alcohol and does not directly enter into the formation of butylene glycol. This is in agreement with the results obtained by Hammer in the case of most of the aldehydes tried with *S. liquefaciens*.

Propionic acid apparently produces the same effect as acetic acid, i.e., causes an increase in butylene glycol, a reduction in the yield of gaseous hydrogen and is itself reduced. It is, however, not attacked quite as vigorously as acetic acid. Propionic acid, though reduced, does not itself directly enter into the formation of butylene glycol. By being reduced to propyl alcohol it causes a reduction in ethyl alcohol and diverts some of the two-carbon compound which ordinarily would go to ethyl alcohol to butylene glycol or acetic acid. These results have been confirmed by repeated experiments. These data indicate that acetic acid may act similarly when added, i.e., be reduced to ethyl alcohol, cause a decrease in gaseous hydrogen and an increase in butylene glycol without itself being transformed to the glycol. When conditions are optimum for the reduction of acetic and the formation of butylene glycol, acetic acid may not be formed.

Fumaric acid was used to determine whether the results were merely the effect of an added hydrogen acceptor. According to Barker (1936), for each mole of fumaric acid oxidized by *Aerobacter aerogenes* another acts as a hydrogen acceptor to yield succinic acid. Fumaric acid has no effect on the formation of butylene glycol. It causes a slight decrease in hydrogen and is itself reduced to succinic acid. However, more succinic acid is formed than can be accounted for by the fumaric added; the decrease in ethyl alcohol accounts for the added increase in succinic. Succinic acid was identified as the p-nitrobenzyl ester.

In several experiments butyric acid has not been found to act as a hydrogen acceptor.

SUMMARY AND CONCLUSIONS

Acetic and propionic acids, when added to glucose fermentations by *Aerobacter indologenes*, are reduced, resulting in an increase in butylene glycol and a decrease in gaseous hydrogen. Propionic acid is reduced to propyl alcohol.

Acetaldehyde undergoes a Cannizzaro reaction when added to an alkaline glucose fermentation by *Aerobacter indologenes*. When added to a fermentation with a reaction favorable to the formation of butylene glycol, increases are noted in acetylmethylcarbinol and ethyl alcohol and decreases in hydrogen.

Propionaldehyde and butyraldehyde are reduced to the corresponding alcohols with some of the butyraldehyde going to butyric acid. No decrease in hydrogen is observed but slight increases in carbon dioxide and the formation of small amounts of succinic acid with decreases in ethyl alcohol are noted.

Fumaric acid is reduced to succinic acid and some of the glucose carbon is also diverted to form succinic at the expense of the ethyl alcohol.

Acetaldehyde is not coupled to form acetylmethylcarbinol by *Aerobacter* at a pH high enough to prevent formation of the latter from glucose.

Since propionic acid produces an effect similar to acetic acid and does not result in the formation of a higher homologous glycol it is doubtful whether acetic acid is a necessary intermediate in the formation of butylene glycol.

It is suggested that perhaps synthetic acetaldehyde, when added, is coupled with some other intermediate than acetaldehyde during the formation of acetylmethylcarbinol.

The mechanism of formation of acetylmethylcarbinol by *Aerobacter* is still an open question.

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