INFLUENCE OF pH ON THE DISSIMILATION OF GLUCOSE BY AEROBACTER INDOLOGENES[1]

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Osburn, Brown and Werkman (1937) observed a critical pH level in the dissimilation of glucose by *Clostridium butylicum* near 6.3. Above this level butyl alcohol and isopropyl alcohol were not formed. The significance of this critical pH was attributed to the fact that in mixtures of acetic (or butyric) acid and its sodium salt below 6.3 there will be "free" acid, i.e., acid which can be distilled from solution and can be converted into alcohols by *C. butylicum*.

This concept of critical pH is applied to the dissimilation of glucose by *Aerobacter indologenes* in the present investigation, particularly with reference to the findings of Reynolds and Werkman (1937), that when sufficient acetic acid is present in a glucose dissimilation by *A. indologenes*, no gaseous hydrogen is evolved and the acetic acid disappearing in the medium is accounted for as 2,3-butylene glycol + acetylmethylcarbinol. Their conclusions were to the effect that acetic acid occurring in the fermentation was transformed into 2,3-butylene glycol + acetylmethylcarbinol.

The principle of critical pH probably has a general application to cellular metabolism. Although pH 6.3 was found to be the critical level in the case of *C. butylicum* in converting butyric and acetic acids into corresponding alcohols, it is likely that each organism will show several such levels which will be determined by the dissociation of the substance subject to change. Furthermore, as pointed out by Osburn and Werkman (1937), the change

in metabolism does not occur sharply at pH 6.3, but the effect is gradual and extends over a relatively narrow range.

The dissimilation of glucose by *A. indologenes* at pH levels below and above 6.3 has been studied in the present work. The results are of particular significance for an understanding of bacterial dissimilation as well as having certain practical implications in procedures involving the presence of acetylmethylcarbinol. In the latter respect, reference may be made to the Voges-Proskauer test and the formation of acetylmethylcarbinol in butter cultures.

In the work of Reynolds and Werkman (1937) no mention of a critical pH level at which the mechanism of dissimilation changes its nature, is made. In the present investigation the significance of levels above and below pH 6.3 is shown in the dissimilation of glucose by *A. indologenes*. The results indicate that the gas ratio of carbon dioxide to hydrogen, the occurrence of acetylmethylcarbinol and 2,3-butylene glycol and the amounts of acetic and formic acids are dependent on the pH of the fermentation and that the range near pH 6.3 is a critical level; below this range, the course of dissimilation is markedly different from that above.

METHODS

Glucose plus acetate fermentations were conducted in 4-liter Erlenmeyer flasks fitted with proper openings for removal of gas, taking of samples, and addition of alkali. Brom-thymol-blue was used as an indicator and the pH maintained by adding normal sodium hydroxide.

The medium consisted of glucose 2 per cent, ammonium sulfate 0.3 per cent, peptone 0.5 per cent, sodium acetate 0.6 per cent and 0.1 M dipotassium phosphate. It is of interest that distilled water was not suitable for making up the medium. Tap water provided excellent growth. When Speakman's salts in distilled water replaced the tap water, growth was not nearly as good. When tap water was evaporated and the residue ashed, addition of the latter provided growth equal to that in the tap water medium. Apparently the essential constituent is inorganic.

The pH was adjusted with sulfuric acid and the medium sterilized at 20 pounds pressure for 30 minutes. Glucose was sterilized separately. Incubation was at 30°C.

Carbon dioxide was collected in Bowen potash bulbs in a drying train. Residual carbon dioxide was determined on a sample collected in alkali which was acidified and refluxed in a stream of CO_2-free air. The carbon dioxide was collected and weighed in a Bowen bulb.

Hydrogen was determined by continuous combustion over heated copper oxide and weighing the water formed. Oxygen-free nitrogen was continuously led through the flasks to remove gases.

An aliquot part of the fermented medium was made acid to congo red and distilled to half volume. The distillate was neutralized to phenolphthalein, again distilled and the alcohol determined on this distillate. The two residues were combined and steam distilled to recover the volatile acids. Total volatile acidity was determined on this distillate by titration. The formic acid was determined according to Auerbach and Zeglin (1922), and the acetic acid obtained by difference. In the presence of acetylmethylcarbinol it was necessary to neutralize the volatile acid distillate, evaporate to small volume and remove at least six volumes by alkaline steam distillation to avoid interference with the determination of formic acid.

Ethyl alcohol was determined on an aliquot part of the neutral volatile fraction by oxidation with a sulphuric acid-potassium dichromate mixture in a closed flask on the steam bath. The mixture was then steam distilled and the acetic acid determined. The alcohol determination was corrected for the acetic acid originating from the acetylmethylcarbinol.

Acetylmethylcarbinol was determined on an aliquot part of the fermented medium according to Stahly and Werkman (1935).

2,3-Butylene glycol was steam distilled from a sample of the medium from which the sugar had been removed by the copper-lime technique of Hewitt (1931); 13 volumes or more were collected from a constant volume of 20 ml. which contained 25 grams of $MgSO_4 \cdot 7H_2O$. Butylene glycol was determined on an

aliquot part of the distillate by oxidation with periodic acid and directly distilling the acetaldehyde into a solution of sodium bisulphite. The aldehyde was determined by titration with iodine.

The lactic acid was determined on the residue according to Friedemann and Graesser (1933).

The experiments with cell suspensions were conducted in Erlenmeyer flasks containing 300 ml. of 0.1 M phosphate with substrate, adjusted to the desired pH. Carbon dioxide and hydrogen were collected in bottles containing alkali; the former was absorbed and determined on an aliquot part, the latter was determined by volume displacement. The hydrogen evolved was in contact with the medium and was available for reduction of the acetic acid. The inoculum consisted of about 5 grams of cell paste per 300 ml. of medium. Air was displaced from the flasks with nitrogen when the fermentations were started. After four days at 30°C. the flasks were removed and contents analyzed according to the procedure used in the glucose ferementations.

<center>EXPERIMENTAL</center>

In tables 1 and 2 are given results of typical experiments in which the influence of pH on the mechanism of dissimilation of glucose by A. indologenes is shown. In the fermentation kept above pH 6.3 (near 7.0) the yields of carbon dioxide and 2,3-butylene glycol are greatly suppressed as compared with an acid fermentation. Acetic and formic acids accumulate and added acetic acid is not attacked. The quantitative relationships of the products are quite different from those in a dissimilation occurring below pH 6.3. Here, acetic acid, even that added, disappears rapidly with the simultaneous occurrence of the 4-carbon compounds, acetylmethylcarbinol and 2,3-butylene glycol (fig. 1); also the production of carbon dioxide is large. Whether the failure of acetic acid to act as an intermediate in alkaline fermentation is due only to a lack of free acid or a dearth of available hydrogen or both, is not clear. Should the presence of formic acid be the result of a synthesis from carbon dioxide and hydrogen, the failure of acetic acid reduction probably results from the utilization of hydrogen in the reduction of carbon

TABLE 1

Fermentation of glucose plus added acetic acid by Aerobacter indologenes.
pH maintained above 6.3

TIME	SUGAR FER-MENTED	CO_2	H_2	FORMIC ACID	ACETIC ACID	LACTIC ACID	ACETYLMETHYLCAR-BINOL	2,3-BUTYLENE-GLYCOL	ETHYL ALCOHOL	2,3-BUTYLENEGLYCOL + ACETYLMETHYL-CARBINOL	CARBON RECOVERY	O/R INDEX
hours											per cent	
0	0				25.77							
16	19.91	15.03		19.15	37.65	2.72	0.24	9.35	15.20	9.59	108	0.89
20	50.90	33.90		49.90	47.70	2.76	1.27	12.50	36.70	13.77	89	1.06
23½	89.30	51.00		74.00	96.05	7.10	1.50	15.60	64.60	17.10	92	0.97
31½	113.10	58.5	2.26	95.00	109.00	5.85	0	22.40	74.80	22.40	86.3	0.97
75	113.10	60.24	9.26	95.00	109.10	6.35	0	22.30	75.60	22.30	87	0.95

Products in millimoles per liter.

TABLE 2

Fermentation of glucose plus added acetic acid by Aerobacter indologenes at
pH 6.3 or below

TIME	SUGAR FER-MENTED	CO_2	H_2	FORMIC ACID	ACETIC ACID	LACTIC ACID	ACETYLMETHYLCAR-BINOL	2,3-BUTYLENE-GLYCOL	ETHYL ALCOHOL	2,3-BUTYLENEGLYCOL + ACETYLMETHYL-CARBINOL	CARBON RECOVERY	O/R INDEX
hours											per cent	
0	0				24.66							
24	35.93	59.59		10.40	11.84		3.64	31.40	20.8	35.04	99	0.92
29	48.73	74.57		10.20	7.00	4.76	4.30	42.00	27.16	46.30	102	0.89
33	59.13	86.52		10.08	4.80	2.28	4.64	45.50	31.46	50.14	95	0.88
48	96.98	132.69	1.24	19.72	5.31	2.20	2.20	70.00	51.54	72.20	90	0.87
96	111.3	215.66	17.69	12.20	3.35	2.46	1.05	82.00	58.40	83.05	96	1.12
168	111.3	225.66	29.07	1.35	4.95	1.78	2.36	82.20	55.80	84.56	96.5	1.15
264	111.3	230.74	30.83	1.26	6.00	2.70	0.72	87.20	60.02	87.92	100	1.14

Products in millimoles per liter.

dioxide rather than acetic acid.   It is to be noted that there was some carbon dioxide and glycol formed in the alkaline fermentation, probably owing to the difficulty of holding the medium alkaline as at times it became sufficiently acid to allow the formation of these products.   In subsequent experiments conducted at pH 7.0 or above, the formation of 2,3-butylene glycol was diminished to a still greater extent—from a normal of approximately 70 mM to 2 mM or less.   The accumulation of acetic
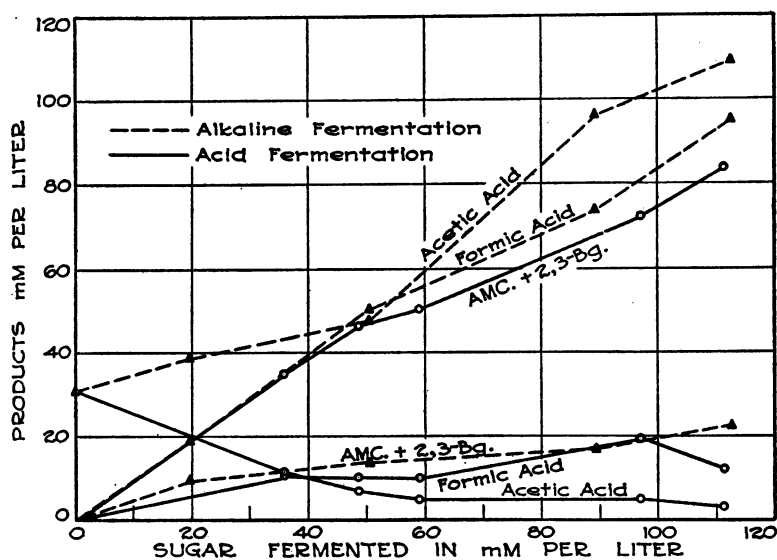

FIG. 1.

acid demonstrates its necessity in an available form for the formation of acetylmethylcarbinol and 2,3-butylene glycol.   Incomplete carbon recoveries with the alkaline fermentations suggest that some undetermined product is formed.

It is interesting to note the influence of pH on the gas ratio which varies within large limits.   When the pH is maintained below 6.3 and sufficient acetate added, the production of hydrogen is completely suppressed and the carbon dioxide formation is large, whereas, in an alkaline medium the production of both

carbon dioxide and hydrogen can be practically prevented. Under the usual conditions of glucose dissimilation the pH drops within the vicinity of 6.0–5.5 but insufficient acetic acid is present to prevent the liberation of hydrogen and a $H_2:CO_2$ ratio of 0.3–0.5 is obtained.

As the fermentation nears completion, the pH shows a reversion owing to the conversion of the acetate ion into neutral compounds.

In regard to the source of hydrogen used in the reduction of acetic acid, it is possible that this is furnished by the dehydrogenation of formic acid. However, using heavy cell suspensions, it was impossible to demonstrate the conversion of acetic acid to acetylmethylcarbinol or 2,3-butylene glycol in the presence of formic acid as H_2-donor (table 3). The fermentations were

TABLE 3

Fermentation of acetic and formic acids by cell suspensions of Aerobacter indologenes in acid and alkaline medium

REACTION OF MEDIUM	ACETIC ACID ADDED	FORMIC ACID ADDED	ACETIC ACID	FORMIC ACID	CARBON DIOXIDE	HYDRO-GEN	FORMIC ACID FER-MENTED
pH 6.0–6.3	112.25	137.76	112.35	72.85	62.20	60.56	64.91
pH 7.0–7.3	111.53	136.90	113.30	121.16	12.40	13.38	15.74

Voges-Proskauer test negative. Products in millimoles per liter.

conducted at pH levels of substantially 6.0–6.2 and at 7.0. It will be observed that the acetic acid was recovered quantitatively in each case and that the formic acid broken down was accounted for quantitatively by equimolar amounts of carbon dioxide and hydrogen. It is also of significance that the formic acid decomposed was about four times greater in an acid than alkaline medium. This finding supports in part, the results from a glucose fermentation under the same conditions. The hydrogen from formic acid did not reduce acetic acid under these conditions. However, in the presence of glucose plus acetic acid the yield of hydrogen is greatly suppressed. If formic acid is the only hydrogen-yielding intermediate it is difficult to account for this behavior which suggests that hydrogen from some other source

is necessary for the conversion of acetic acid to acetylmethyl-carbinol. Formic acid hydrogen is able to reduce acetylmethyl-carbinol to 2,3-butylene glycol by *Escherichia coli* (table 4).

The effect of pH on the action of the formic hydrogenlyase is important. Krebs (1937) found that *E. coli* did not form lactic acid from pyruvic acid anaerobically when the pH was between 6.6–8.0. However, if the pH was lowered to 5.4, considerable lactic acid and carbon dioxide were produced. In an alkaline medium. formic and acetic acids were the only final products of pyruvic breakdown. Woods (1936) found in the case of *E. coli* that an alkaline reaction is necessary for a synthesis of formic acid from carbon dioxide and hydrogen. We have

TABLE 4

Reduction of acetylmethylcarbinol by cell suspensions of Escherichia coli and formic acid

	ACETYL-METHYL-CARBINOL	CARBON DIOXIDE	HYDRO-GEN	FORMIC ACID	ACETIC ACID	2,3-BU-TYLENE-GLYCOL	CARBON RECOV-ERY
							per cent
Initial.............	54.25			112.77			
Final..............	0	76.31	30.10	39.69	25.73	41.91	102

Products in millimoles per liter.

found that formate in an acid medium is decomposed vigorously. However, at alkaline levels the conditions are relatively more favorable for the synthesis of formic acid than its breakdown. Since formic hydrogenlyase is a reversible system, the retention of carbon dioxide in the medium may tend to drive the reaction toward the synthesis of formate.

SUMMARY AND CONCLUSIONS

A critical pH level has been shown to exist in the dissimilation of glucose by *A. indologenes* in the region of 6.3. Fermentation carried out above this level results in an accumulation of acetic and formic acids. The production of hydrogen and carbon dioxide is greatly suppressed and the formation of acetylmethyl-

carbinol and 2,3-butylene glycol may be prevented if care is taken not to allow the pH to drop within the vicinity of 6.3. When the fermentation occurs below pH 6.3, the acetic acid is converted into acetylmethylcarbinol and 2,3-butylene glycol. If sufficient acetic acid is added to an acid fermentation, the production of gaseous hydrogen is prevented.

It is suggested that the acidity and alkalinity of the medium expresses itself by determining the relative hydrogen accepting ability of acetic acid and carbon dioxide. When the medium is alkaline, carbon dioxide is the better hydrogen acceptor resulting in the formation of formic acid; under acid conditions (approximately 6.3 or less) carbon dioxide cannot compete with acetic acid and acetylmethylcarbinol and 2,3-butylene glycol are formed.

For the conversion of acetic acid into neutral compounds, it is important to have both free acetic acid and available hydrogen. Evidence suggests that the general occurrence of formic acid among the final products of bacterial dissimilation of glucose may be the result of a synthesis from carbon dioxide and hydrogen, a fact which would have important implications in formulating schemes of dissimilation particularly with reference to the breakdown of pyruvic acid into formic and acetic acids or acetaldehyde and carbon dioxide.

The results point clearly to the fact that the gas ratio of $(H_2:CO_2)$ 0.5 is fortuitous. This generally accepted ratio of 0.5 is the result of the conditions under which the determination is made and may vary within wide limits. Review of the literature dealing with quantitative experiments shows that the ratio is usually near 0.3, indicating a marked utilization of hydrogen for the reduction of acetic acid to 2,3-butylene glycol.

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