THE DISSIMILATION OF GLYCEROL BY COLI-AEROGENES INTERMEDIATES

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Received for publication November 20, 1939

In 1928 Braak gave a detailed account of the dissimilation of glycerol by coli-aerogenes bacteria. In addition to *Escherichia* and *Aerobacter*, the fermentation of glycerol by two organisms of the intermediate group was studied. The intermediates were considered unique in their fermentative properties because they were able to dissimilate glycerol in an inorganic medium under anaerobic conditions, and apparently used glycerol itself as a hydrogen acceptor. In this respect the intermediates differed from *Escherichia* and *Aerobacter* which required the presence of peptone, yeast extract, oxygen or other hydrogen acceptor in order to attack glycerol under similar conditions. The utilization of glycerol as a hydrogen acceptor resulted in the formation of trimethyleneglycol. It is interesting to note that the dissimilations reported by Braak for *Escherichia* and *Aerobacter* were practically identical. Recently Mickelson and Werkman (1940) have shown that four strains of *Aerobacter*, contrary to the findings of Braak, fermented glycerol anaerobically in a mineral-salts glycerol medium, forming trimethyleneglycol.

The fermentation of glycerol with the production of trimethyleneglycol has been further studied in this laboratory by Gillen (1930), Davis (1931) and Tarnutzer (1932).

In recognition of Freund, who first observed the formation of trimethyleneglycol from glycerol by bacteria, Braak named his organisms *Bacterium freundii.*

Werkman and Gillen (1932) proposed the name *Citrobacter* for a new genus to include those coliform organisms intermediate between *Escherichia* and *Aerobacter.* Six species were described all of which formed trimethyleneglycol from glycerol. *Citrobacter freundii* was designated as the type species of the new genus. However, the genus was not to be limited to those organisms which formed trimethyleneglycol.

Previous quantitative work on the fermentation of glycerol by *Citrobacter* has been complicated by difficulty in obtaining complete recovery of the carbon.

The purpose of this investigation was to contribute to our knowledge of the intermediate mechanism of the dissimilation of glycerol by organisms of the coli-aerogenes intermediates.

EXPERIMENTAL

A medium of 2 per cent glycerol, 1 per cent phosphate buffer (pH 6.6–6.8) and 0.3 per cent ammonium sulphate in tap water was used. It was found desirable to have the pH of the medium slightly acid in order to initiate rapid growth.

Fermentations were conducted anaerobically at 30°C. *Citrobacter freundii* and two other strains of the methyl-red-positive, citrate-positive, Voges-Proskauer-negative section of the coli-aerogenes group were studied. The fermentation flasks were fitted with burettes through which CO_2-free N sodium hydroxide was added to maintain a satisfactory pH.

The methods of analysis were those reported in a previous paper on the fermentation of glycerol by *Aerobacter* (Mickelson and Werkman, 1940). Acrolein added was determined by fixing the aldehyde with bisulphite and titrating the bound bisulphite with standard iodine. The acrolein in the fermentation medium was distilled into 1 per cent sodium bisulphite solution, and the bisulphite of the aldehyde complex was titrated in a similar manner. In fermentations to which fumaric acid was added, the sample used for the determination of succinic acid was treated as described by Brewer and Werkman (1939). This procedure serves to separate fumaric, and other acids oxidized by permanganate, from succinic acid.

Some of Braak's data on *Bacterium freundii,* recalculated as millimoles of product per 100 millimoles of fermented glycerol are given in table 1. In most cases incomplete recoveries of carbon were obtained, perhaps because of incomplete recovery of some of the products by the methods used. In those instances where lowest carbon recoveries were obtained, the oxidation-reduction index shows the greatest shortage of reduced products. For a discussion of oxidation-reduction relations between substrate and products see Johnson, Peterson and Fred (1931). A perfect index equals 1. In view of our results, it is reasonable to suspect that there has been incomplete recovery of ethyl alcohol or trimethyleneglycol or both. At any rate it is apparent that the deficient carbon is a reduced compound.

TABLE 1

Data on dissimilation of glycerol by Bacterium freundii—recalculated from Braak

Products per 100 millimoles of fermented glycerol

ORGANISM	CO_2	H_2	FORMIC ACID	ACETIC ACID	LACTIC ACID	SUCCINIC ACID	ETHYL ALCOHOL	2,3-BUTYLENE GLYCOL	TRIMETHYLENE GLYCOL	O/R INDEX*	PER CENT CARBON
I, Strain 19....	23.00	24.60	3.72	14.40	5.40	3.38	15.20	0	35.70	1.21	74.20
II, Strain 19....	18.95	17.80	3.03	11.72	6.06	4.17	15.15	0	35.20	1.22	72.40
III, Strain 19....	29.10	28.05	10.20	8.65	14.80	2.78	33.00	0	27.20	1.15	87.20
IV, Strain 20....	36.00	37.30	12.70	4.80	12.30	2.19	48.30	0	23.60	1.03	90.50

*1 = a perfect index.

Media: I, 1475 ml. mineral solution, 1.94 per cent glycerol, 1.0 per cent $CaCO_3$; II, 1450 ml. mineral solution, 1.68 per cent glycerol, 1.67 per cent $CaCO_3$; III, 1450 ml. peptone water, 2.06 per cent glycerol, 1.66 per cent $CaCO_3$; IV, 1450 ml. mineral solution, 1.51 per cent glycerol, 1.00 per cent $CaCO_3$.

Since Braak was able to detect small quantities of acrolein in some of his distillates, he suggested that the loss of carbon was due to a polymer of acrolein which escaped determination.

Table 2 shows the results obtained on the fermentation of glycerol by *Citrobacter freundii* and one other strain of *Citrobacter* (no. 46). The recovery of carbon is satisfactory and indications are that there is no carbon lost in an undetermined compound. Since the formation of trimethyleneglycol is a reduction, it was of interest to determine whether a competing hydrogen acceptor would inhibit its formation. Previous work by Tarnutzer (1932) had indicated that the addition of bisulphite and fumaric acid prevented the reduction of glycerol to trimethyleneglycol. The results we have obtained to date do not confirm these findings. Experiment 2 in table 1 shows the effect of adding fumaric acid. The acid was reduced to succinic and the yield of hydrogen decreased but no inhibition of the formation of trimethyleneglycol was apparent.

TABLE 2

Anaerobic dissimilation of glycerol by Citrobacter

Products per 100 millimoles of fermented glycerol

STRAIN OF CITROBACTER	CO_2	H_2	FORMIC ACID	ACETIC ACID	LACTIC ACID	SUCCINIC ACID	ETHYL ALCOHOL	TRIMETHYLENE GLYCOL	ACROLEIN	O/R INDEX	PER CENT CARBON
C. freundii I......	20.0	21.1	18.60	12.30	4.80	7.90	30.90	36.00		1.075	94.1
C. 46 I...........	11.4	12.75	22.55	8.40	12.10	6.40	29.40	37.70		1.035	94.5
C. freundii II.....	25.90	3.52	11.05	12.15	3.56	32.60	28.15	37.90		1.05	99.5
C. freundii III....	7.63	2.50	9.90	9.00	11.20	4.02	7.95	55.60	4.20	0.975	90.8
C. freundii IV....	5.52		8.40	14.80	17.00	3.28	4.15	60.40		0.95	99.0

Control medium: 2.0 per cent glycerol, 0.3 per cent $(NH_4)_2SO_4$, 1 per cent K_2HPO_4, 10 per cent tap water, volume approximately 750 ml.

I, control medium; II, control medium + 24.7 mM fumaric acid; III, control medium with 5 grams $CaCO_3$ instead of K_2HPO_4 and 0.1 per cent $NaHSO_3$; IV, control medium.

The addition of sodium bisulphite to a calcium-carbonate-buffered medium produced an interesting result. Experiment 3 shows an increase in trimethyleneglycol with a reduction in yield of ethyl alcohol and hydrogen. A second experiment showed a similar effect. The addition of bisulphite as the sodium salt when potassium phosphate was used as buffer prevented growth. Apparently calcium is necessary to keep the concentration of soluble sulphite below a toxic level. The exact effect of bisulphite is not clear, however it is apparent that the reduction processes were diverted away from the formation of alcohol to that of trimethyleneglycol. However, later experiments, one of which is shown in table 2, experiment 4, gave high yields of trimethyleneglycol without the presence of bisulphite. The high yields of the glycol in the absence of bisulphite make it doubtful whether the fixative actually was responsible for the increased yield of trimethyleneglycol obtained in its presence.

In the presence of bisulphite considerable acrolein accumulated in the medium. Braak (1928) reported the occurrence of acrolein in distillates from glycerol fermentations by *Bacterium freundii*. Reynolds, Hoehn and Werkman (1939) report the fixation of acrolein in glycerol fermentations by *Citrobacter freundii* with dimedon as a fixative. The use of calcium sulphite caused a fixation of pyruvic acid and acetaldehyde but no acrolein.

On distillation of the bisulphite fermentation liquor a very strong odor of acrolein was apparent. Concentration of the material and preparation of the 2,4-dinitrophenylhydrazone gave a compound which had the melting point of the acrolein derivative (163°C.). When mixed with the 2,4-dinitrophenylhydrazone of pure acrolein the melting point remained unchanged. Since the melting points of the 2,4-dinitrophenylhydrazones of acetaldehyde and acrolein are very close together, a mixed melting point of the acetaldehyde derivative and the acrolein derivative was taken. A mixture of the two derivatives gave a melting point of 145°C. indicating that the unknown derivative was not that of acetaldehyde. The presence of acrolein in fermentations of glycerol suggests that it may be an intermediate and, if so, most likely concurs in the formation of trimethyleneglycol. The formation of trimethyleneglycol from glycerol involves a reduction, essentially the replacement of an OH group by H. This is analogous to the formation of propionic acid from lactic acid by the propionic acid bacteria. Such a reaction can be conveniently schematized by removal of water from the molecule with the formation of a double bond and then hydrogenation of the double bond to form a methylene group instead of a hydroxyl. In such a scheme acrylic acid, in the case of the lactate fermentation by propionic bacteria, and acrolein, in the glycerol fermentation by trimethyleneglycol-forming bacteria, would find useful rôles. In view of these possibilities, the addition of acrolein to glycerol-fermenting cultures of *Citrobacter freundii* was attempted to demonstrate whether it was converted to trimethyleneglycol. The aldehyde was added slowly from a dropping funnel but even in very small quantities, it inhibited fermentation. Several attempts were unsuccessful; van Niel (personal communication) stated that similar attempts to cause the propionic acid bacteria to form propionic acid from acrylic acid were unsuccessful. The apparent toxicity of the acrolein may not be sufficient to discount it completely as an intermediate. In a normal fermentation its presence may be so transitory that a toxic concentration is never attained. However, if acrolein is an intermediate in the formation of trimethyleneglycol, it is of interest that in the presence of sulphite the yields of trimethyleneglycol are not decreased (table 2, experiment 3). The finding of small quantities of the aldehyde in glycerol fermentations by *Citrobacter freundii* to which no fixative has been added and the extreme toxicity which it manifests when added to fermenting cultures might be taken to mean that it is formed in a manner purely incidental to the formation of trimethyleneglycol and actually plays no intermediate rôle.

The influence of sulphite on the formation of trimethyleneglycol is complicated by the fact that in several experiments where no sulphite was added equally high yields of trimethyleneglycol were obtained. Such high yields of the glycol suggest the practicability of the fermentation in preparing trimethyleneglycol. With approximately 60 per cent of the glycerol being reduced in some instances the reduction might be extended even further if the simultaneous oxidations could be carried toward completion, i.e., to a higher state of oxidation.

SUMMARY

The fermentation of glycerol by several species of the genus *Citrobacter* is reported and discussed.

Yields of trimethyleneglycol varying from approximately 30 per cent to 60 per cent of the fermented glycerol have been obtained.

The isolation of acrolein and its possible importance in the fermentation process is discussed.

The effect of the addition of sulphite as a fixative and fumaric acid as a hydrogen acceptor has been determined. Sulphite did not diminish the formation of trimethyleneglycol and in its presence considerable acrolein accumulated. Acrolein was not attacked when added to fermenting glycerol. Fumaric acid, though reduced, did not retard the formation of trimethyleneglycol.

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