X. THE UTILISATION OF CO_2 IN THE DISSIMILATION OF GLYCEROL BY THE PROPIONIC ACID BACTERIA.

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(*Received December 2nd, 1935.*)

REDTENBACHER [1846] was probably the first to study the fermentation of glycerol by the propionic acid bacteria. Largely by accident he obtained a good growth of these organisms in probably impure culture and found propionic acid as substantially the only product. It remained for Van Niel [1928] to confirm the results of Redtenbacher with pure cultures. He found that glycerol was converted quantitatively into propionic acid under anaerobic conditions with no formation of gas. Van Niel suggested that glyceraldehyde is an intermediate product which is reduced to propionic acid. Pett and Wynne [1933, 1] obtained preliminary evidence for the formation of methylglyoxal and of glyceraldehyde (or dihydroxyacetone) from sodium β-glycerophosphate by fermentation with dried propionic acid organisms. These authors indicate that their identifications are not conclusive. Wood and Werkman [1934] using both $CaSO_3$ and dimedon (dimethyldihydroresorcinol) as fixatives isolated and identified propaldehyde from fermentations of glycerol. They suggested that this compound occurs as an intermediary and is subsequently converted into propionic acid. Pyruvic acid has also been identified (unpublished results).

This brief review summarises our knowledge of the dissimilation of glycerol by the propionic acid bacteria. In the present communication it will be shown that propionic acid is not the only product which may be formed and that the dissimilation is more complex than found by previous investigators.

METHODS.

The volatile acids were determined by the partition method of Osburn *et al.* [1933]. Non-volatile acids were extracted with ethyl ether continuously for 12 hours from the residue of steam-distillations which was taken up in anhydrous Na_2SO_4. The lactic acid was determined by the method of Friedemann and Kendall [1929]. The succinic acid was obtained by neutralising the hot solution with $CaCO_3$, filtering and precipitating the calcium succinate in 85% ethyl alcohol. The calcium salt was determined by weight. This acid was also determined by the silver salt method [Moyle, 1924]. The original glycerol was determined by weight. The residual glycerol determination was made on an acidified aliquot part of the medium which was evaporated on a steam-bath to 50–75 ml., neutralised, taken up in plaster of Paris and extracted continuously with acetone for 8 hours. The acetone extract was reduced to 50–75 ml., distilled water added and the acetone removed by further distillation. The glycerol was determined in this preparation by the method of Wagenaar [1911]. The original

[1] Supported in part by a grant from the Industrial Science Research funds of the Iowa State College for the study of the Utilisation of Agricultural By-products.

CO_2 of the medium was calculated equivalent to the weighed quantity of $CaCO_3$ used in the medium. The liberated CO_2 was determined in two ways. It was either absorbed in soda-lime and weighed or it was absorbed in standard alkali and the excess alkali titrated with standard HCl after the addition of an excess of $BaCl_2$ to precipitate the CO_2. The soda-lime was used in a train of 6-inch U-tubes with $CaCl_2$ as the drying agent. When alkali was used it was contained in two bottles connected by a syphon. The first bottle was attached to the fermentation flask; the second was supplied with a soda-lime tube to prevent entrance of CO_2 from the air. The syphon permitted displacement of the alkali as gas was formed during fermentation. The alkali was transferred to a bottle fitted with a bubble spiral-absorber for the collection of residual CO_2. The residual CO_2 was obtained as follows. The fermentation flask was attached to a reflux condenser which led to the soda-lime train or bubble spiral-absorber and the CO_2 was liberated by adding a known volume of dilute H_2SO_4 slightly in excess of that necessary to react with the $CaCO_3$, a slow current of CO_2-free air was passed through and the contents finally brought to the boil. The CO_2 collected *minus* the CO_2 added as $CaCO_3$ equals the CO_2 produced by the fermentation. The method proved accurate with known quantities of $CaCO_3$.

The media used were as follows. The experiments of Table II were carried out with 700 ml. of medium consisting of glycerol 3%, $CaCO_3$ 2% and yeast extract (Difco) 0·4% in 1 litre Erlenmeyer flasks. These fermentations were maintained anaerobic by continuously bubbling oxygen-free nitrogen through the fermenting medium. The CO_2 was absorbed in soda-lime. The results shown in Table III were obtained with 800 ml. of medium containing glycerol 2%, $CaCO_3$ 1·14% and yeast extract 0·4%. Conditions were made anaerobic by displacing the air in and above the medium with nitrogen immediately after inoculation. The CO_2 was collected in alkali. Constituents of the media were sterilised separately at 20 lb. pressure for half an hour and mixed at the time of inoculation. 3–5-day cultures grown in a yeast extract medium with 0·5% glucose and equivalent to 5% by volume were used as inoculum. The flasks were shaken twice daily to aid the buffer action of the carbonate. Purity of cultures before and after fermentation was established by the Kopeloff-Gram stain, absence of growth on aerobic slants and the zone of growth and colony formation in agar shakes. Incubation was at 37°, for the fermentations of Table II and at 30° for those of Table III. The incubation period was 35 days.

The cultures are identified in Table I. Complete descriptions of the species are given by Werkman and Brown [1933].

Experimental.

Results are expressed as millimoles per litre of medium in Table II and per 100 millimoles of fermented glycerol in Table III. The carbon recovery (Table III) is calculated first on the basis of the glycerol fermented and CO_2 utilised and

Table I. *Identification of cultures.*

Culture no.	Species	Culture no. used by other investigators*
11W	*P. petersonii*	*Sherman*—22, Van Niel—20
15W	*P. technicum*	*Sherman*—10
34W	*P. arabinosum*	*Hitchner*—61
49W	*P. pentosaceum*	Foote, *Fred* and Peterson—11 Van Niel—4
52W	*P. shermanii*	Foote, *Fred* and Peterson—19
4875	*P. pentosaceum*	American Type Culture Collection

* Culture received from investigator whose name is italicised.

Table II. *Dissimilation of glycerol by propionic acid bacteria.*

Culture no.	Products per litre: Propionic acid mM.	Acetic acid mM.	Succinic acid mM.	CO_2 utilised mM.
4875	101·7	6·5	32·2	46·6
34 W	137·6	2·8	28·8	43·0
49 W	128·0	7·7	19·1	25·6
49 W	168·9	9·3	130·0	—

Table III. *Dissimilation of glycerol by propionic acid bacteria.*

Culture no.	Glycerol fermented per litre mM.	CO_2 utilised per 100 mM. of fermented glycerol mM.	Products per 100 mM. of fermented glycerol: Propionic acid mM.	Acetic acid mM.	Succinic acid‡ mM.	Carbon recovery: Basis-glycerol *plus* CO_2 %	Basis-glycerol only %	Oxidation-reduction index: Basis-glycerol *plus* CO_2	Basis-glycerol only
49W	212·6	37·7	55·8	2·9	42·1	101·2	114·0	1·081	2·550
34W	209·0	43·2	59·3	2·0	34·5	93·1	106·6	0·925	2·270
52W*	112·0	20·0	78·4	5·9	8·7	94·6	101·0	0·918	1·386
11W†	218·4	1·1	89·3	2·6	3·9	96·5	96·8	1·135	1·162
15W	176·4	12·3	78·4	5·8	7·8	89·1	92·6	1·047	1·376

* 7·0 mM. of lactic acid produced per 100 mM. of fermented glycerol.
† 0·5 mM. of lactic acid produced per 100 mM. of fermented glycerol.
‡ Succinic acid identified by melting-point and mixed melting-point.

secondly on the basis of glycerol alone. The redox indices (Table III) are calculated on the same bases. The redox (oxidation-reduction) index of a fermentation is obtained by (1) multiplying the mM. of each product by its respective oxidation or reduction value based on water as zero (H_2 is equivalent to one atom of oxygen) [*cf.* Johnson *et al.*, 1931]; (2) dividing the sum of oxidation values by reduction values. A perfect balance is represented by an index of 1·0. Propionic acid has a reduction value of 1, succinic an oxidation value 1 and acetic acid is neutral. Glycerol has a reduction value of 1, therefore its utilisation is represented as an oxidation and the glycerol fermented is given an oxidation value 1. Likewise, the utilisation of CO_2, an oxidised compound, represents a reduction and its reduction value is 2.

The glycerol fermented was not determined in Table II. The quantity of non-reducing compounds was not sufficient to influence the fermentation balances.

Discussion.

The most significant fact shown by the data is the apparent utilisation of CO_2 by the propionic acid bacteria. This was evident, since the CO_2 at the conclusion of the fermentation was not equivalent to that of the original medium in the form of $CaCO_3$. This observation has been substantiated by two types of calculation of especial value, *i.e.* carbon recovery and redox index. If CO_2 is utilised, and is in turn (after synthesis) dissimilated, then calculations based on the assumption that glycerol is the sole source of carbon, should show an excess of products, *i.e.* the calculated recovery of carbon will exceed 100%. Table III shows that this occurred and that calculations based on glycerol *plus* CO_2 are acceptable. The calculation of carbon recovery is not in all cases entirely satisfactory proof of CO_2 utilisation, but the oxidation-reduction balance is convincing. CO_2 contains but one carbon and requires a large utilisation to show a

detectable change in the carbon balance; in the oxidation-reduction balance the CO_2 is highly oxidised and therefore has a marked effect. The data show that results calculated on the basis of glycerol *plus* CO_2 are reasonable and acceptable. The fact that the chemical analysis shows a decrease of CO_2 is perhaps proof enough of CO_2 utilisation. However, the carbon and oxidation-reduction balances furnish additional evidence.

The CO_2 analysis has been checked by two different methods, and four species of *Propionibacterium* of known purity, each received from a different source, have shown a distinct utilisation of CO_2 under the conditions of our experiments. Every culture examined with the possible exception of 11W showed ability to utilise CO_2. This behaviour is probably characteristic of the genus *Propionibacterium*.

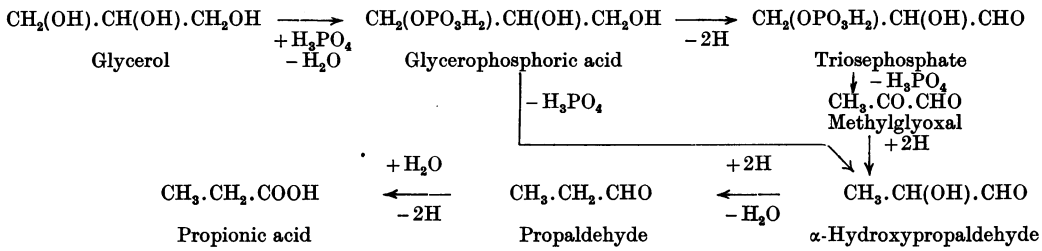
This observation requires a reinterpretation of previous results. Investigators have not considered the possibility of CO_2 utilisation in constructing schemes of dissimilation. If one considers the limited number of bacteria which have been shown to utilise CO_2 and also that such forms differ markedly from the propionic acid bacteria, failure to consider the possibility of CO_2 utilisation may be understood. It is of interest that a number of investigators have found that growth of bacteria in general is inhibited in the absence of CO_2. Rockwell and Highberger [1927] suggested that CO_2 is utilised by bacteria and expressed the opinion that it is the only direct source of carbon. It is possible that the utilisation of CO_2 as a source of carbon may not be limited to the small group of bacteria now recognised. Since most bacteria produce CO_2 during their growth it is difficult to determine whether they utilise CO_2. In the present case, the propionic acid bacteria utilised more CO_2 than was produced and thus offered direct evidence of CO_2 consumption.

One important problem, which requires consideration in relation to the data presented, is the mechanism of succinic acid formation. A number of investigators working particularly with yeast and fungi [Butkewitsch and Federoff, 1930; Wieland and Sonderhoff, 1933] have reported that succinic acid is formed by a condensation of two molecules of acetic acid. Virtanen [1925; 1934] and Virtanen and Karstrom [1931], however, have suggested that the propionic acid bacteria produce succinic acid from glucose by a 4- and 2-carbon cleavage of the hexose molecule. Virtanen's proposal was prompted by the observation that the propionic acid bacteria in the presence of toluene form succinic and acetic acids from glucose with no gas. This observation appeared incompatible with schemes involving a 3-carbon cleavage and the formation of 2-carbon compounds by a 2- and 1-carbon cleavage. The absence of CO_2 or other 1-carbon compounds appeared conclusive proof against such a scheme. However, the present evidence of the utilisation of CO_2 by the propionic acid bacteria leaves no reason to assume that the 1-carbon compounds should equal the sum of the 2-carbon compounds. It is necessary in the light of our present knowledge to leave open the possibility of a 4- and 2-carbon cleavage.

The mechanism of fermentation (Tables II and III) is of interest but at present must remain largely speculative. The scheme of Van Niel is not complete since it does not include succinic acid. His suggestion that the succinic acid is formed from the yeast extract is excluded in these fermentations as in some a quantity of succinic acid was obtained which exceeded the total quantity of yeast extract used in the medium. The formation of the 4-carbon compound (succinic acid) from the 3-carbon compound (glycerol) is proof of synthesis. It is possible that the synthesis yields a 6-carbon compound which is dissimilated to succinic acid by a 4- and 2-carbon cleavage. However, such a scheme for the

glycerol fermentation would require a synthesis from a 2-carbon compound since the 2-carbon and 4-carbon compounds do not occur in equivalent quantities. It seems more probable that part of the glycerol is dissimilated to 2- and 1-carbon compounds and that the 1-carbon compound is completely utilised in the synthesis of a compound which is subsequently fermented whilst the 2-carbon compound, probably acetic acid, condenses to yield succinic acid. The evidence presented justifies the assumptions relative to the utilisation of the 1-carbon compound. With regard to the formation of succinic acid by a condensation of 2-carbon compounds more complete evidence will be presented elsewhere. The results obtained by different methods indicate that the propionic acid bacteria can produce succinic acid by a condensation of 2-carbon compounds and it is probable that the condensation involves acetic acid. The formation of succinic acid from glycerol is indirect support of such a mechanism although the possibility cannot be disregarded that other reactions are occurring. It is possible that the succinic acid is formed in more than one way.

Explanation of the formation of propionic acid also offers difficulties. Wood and Werkman [1934] furnished some information by their isolation of propaldehyde but the question arises as to the manner of formation of the propaldehyde. The investigations of Embden *et al.* [1933] and Meyerhof and Kiessling [1933] have emphasised the importance of glycerophosphoric acid and phosphoglyceric acid in the carbohydrate metabolism of yeast and muscle tissue. This suggests the possible rôle of these compounds in the dissimilation of glycerol by the propionic acid bacteria. In the schemes proposed by Embden and Meyerhof the triosephosphate is converted into phosphoglyceric acid. To obtain propaldehyde following such a conversion the carboxyl group would have to be reduced to a carbonyl group. It is doubtful whether the propionic acid bacteria can bring about such a conversion. A more probable series of reactions leading to the formation of propaldehyde and propionic acid is that shown below.



Mechanism of the dissimilation of glycerol to propionaldehyde and propionic acid.

Pett and Wynne [1933, 2] have found that the propionic acid bacteria can dephosphorylate glycerophosphoric acid and also [1933, 1] obtained preliminary evidence of the occurrence of methylglyoxal and glyceraldehyde (or dihydroxyacetone). Reduction of methylglyoxal to propaldehyde might reasonably be expected, for the reduction of pyruvic acid to propionic acid is readily accomplished by these organisms. The proposed scheme involving methylglyoxal appears from our present knowledge to be a logical means of obtaining propaldehyde although other reactions cannot be excluded. For example, the glycerophosphoric acid may go directly to α-hydroxypropaldehyde.

Phosphoglyceric acid may not play a rôle in the formation of propaldehyde although it may be an important intermediary in the fermentation yielding pyruvic acid and subsequently acetic acid, CO_2 and propionic acid. There is no

reason apparent why methylglyoxal and phosphoglyceric acid cannot occur in the same fermentation or why a compound cannot be formed by more than one series of reactions.

It is not clear why our fermentations gave results so different from those of Van Niel [1928]. His data in which $CaCO_3$ was used as a buffer give no information relative to the CO_2 but show that the dissimilation was not the same, with the possible exception of that obtained with culture 11W. Van Niel accounted quantitatively for his fermented glycerol as propionic acid. The action of CO_2 as a hydrogen acceptor is made evident in our fermentations by the production of the oxidised product succinic acid. It is apparent that a large CO_2 utilisation is accompanied by a greater production of succinic acid or some other oxidised compound.

Summary.

An investigation has been made of the dissimilation of glycerol by bacteria of the genus *Propionibacterium* and the products have been determined quantitatively. The results obtained support the following conclusions.

1. CO_2 obtained from $CaCO_3$ is utilised by the propionic acid bacteria in their fermentation of glycerol.

2. Propionic, acetic, succinic and occasionally lactic acids are produced from the glycerol. CO_2 acts as a hydrogen acceptor permitting the formation of oxidised products.

3. The utilisation of CO_2 by the propionic acid bacteria necessitates further interpretation of data and places in question the evidence for the 4- and 2-carbon cleavage.

4. The formation of succinic acid from glycerol offers indirect evidence of its formation by a condensation of 2-carbon compounds.

5. The mechanism of glycerol dissimilation is shown to be complex, involving synthetic reactions with subsequent fermentation of a synthesised product.

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