LXXXVIII. MECHANISM OF GLUCOSE DISSIMILATION BY THE PROPIONIC ACID BACTERIA.

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THE first work of importance on the chemistry of the propionic acid fermentation was that of Fitz [1878] who suggested that the dissimilation of lactic acid occurs according to equation (1).

$$3CH_3.CHOH.COOH \rightarrow 2CH_3.CH_2.COOH + CH_3.COOH + CO_2 + H_2O \quad ......(1).$$

Virtanen [1923; 1925], Virtanen and Karström [1931] and Van Niel [1928] supposed that glucose after preliminary phosphorylation is converted into two molecules of the C_3 type, one being oxidised to acetic acid and CO_2 whilst two molecules are reduced to propionic acid. Van Niel suggested that pyruvic acid is an intermediary, dissimilated according to equation (2).

$$CH_3.CO.COOH + H_2O \rightarrow CH_3.C(OH)_2.COOH \rightarrow CH_3.COOH + CO_2 + 2H \quad ......(2).$$

Virtanen [1923] first noted the formation of succinic acid in glucose fermentations and suggested that it was formed by a 4- and 2-carbon cleavage (equation 3).

$$C_6H_{12}O_6 \rightarrow COOH.CH_2.CH_2.COOH + CH_3.CHO + H_2O \quad ......(3).$$
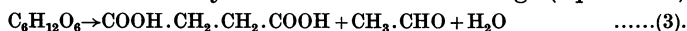
A number of the proposed intermediate compounds have been isolated or detected. Foote *et al.* [1930] and Fromageot and Tatum [1933] found lactic acid, Virtanen and Karström [1931] hexosemonophosphate, Pett and Wynne [1933] methylglyoxal and Wood and Werkman [1934, 1] pyruvic acid. Wood and Werkman [1934, 2] isolated propionaldehyde and offered more definite information on the mechanism of propionic acid formation (equation 4).

$$CH_3.CH_2.COH + H_2O \rightarrow CH_3.CH_2.CH(OH)_2 \rightarrow CH_3.CH_2.COOH + 2H \quad ......(4).$$

They [1934, 3] detected a non-reducing material and studied the mechanism of the fermentation.

The present investigation deals with the mechanism of glucose dissimilation by several species of *Propionibacterium*. Results have been obtained which show the dissimilation to be more complex than that proposed by previous investigators.

METHODS.

The analytical procedure was similar to that described by Wood and Werkman [1936]. The sugar determinations were made before and after fermentation according to the method of Munsen and Walker [1906]. Non-reducing sugars were determined after hydrolysing with 2·25 % HCl for 2·5 hours and determining the increase in reducing sugars. The CO_2 was absorbed in soda-lime or in standard NaOH and the volatile acids and non-volatile acids were determined as previously described. Purity of the cultures was verified. Constituents of the medium were sterilised separately and mixed at the time of inoculation. The inoculum was equivalent to 5 % by volume and consisted of cultures grown in a medium containing 0·5 % glucose. Fermentations were conducted under nitrogen in litre flasks. All cultures used have been identified and described by Wood and Werkman [1936] and Werkman and Brown [1933]. Culture numbers and species

are as follows: 52W and 48W, *P. shermanii*; 49W and 36W, *P. pentosaceum*; 34W, *P. arabinosum* and 11W, *P. petersonii*. Culture 48W is culture 5 of Foote *et al.* [1930]. The results are calculated as described by Wood and Werkman [1936]. The non-reducing material is calculated as a 6-carbon sugar.

EXPERIMENTAL.

The results presented in Table II are unlike others reported in the literature and schemes suggested by previous investigators do not meet the requirements of these data. The ratios of propionic acid to acetic acid vary from 2·13 to 14·72;

Table I. *Conditions of experiments shown in Table II.*

Culture and fermentation no.	Tempera- ture of in- cubation ° C.	Period of incubation days	Age of inoculum days	Medium			Volume of medium fermented ml.
				Glucose %	CaCO₃ %	Bacto-yeast extract %	
34 W₁	37	18	5	3·0	1·4	0·5	700
34 W₂	30	18	5	2·1	1·0	0·35	990
49 W₃	37	15	5	3·0	1·4	0·5	700
49 W₄	30	15	5	3·0	1·4	0·5	700
36 W₅	37	24	5	3·0	1·4	0·4	700
48 W₆	37	24	5	3·0	1·4	0·4	700
52 W₇	30	40	2	3·0	1·5	0·4	800
52 W₈	30	40	2	3·0	1·5	0·4	800
11 W₉	30	12	5	2·4	2·5	0·5	800
11 W₁₀	30	12	5	2·4	2·0	0·7	800

Table II. *Dissimilation of glucose by the propionic acid bacteria.*

Culture and fer- mentation no.	Glucose fermented per litre m.mol.	Quantities produced per 100 m.mol. of fermented glucose					Ratio CO₂/ acetic acid	Ratio propionic/ acetic acid	C re- covered %	Redox index
		Propionic acid m.mol.	Acetic acid m.mol.	CO₂ m.mol.	Succinic acid m.mol.	Non-re- ducing material m.mol.				
34 W₁	145·3	139·1	17·8	63·6	13·2	2·8	3·59	7·82	97·6	1·01
34 W₂	109·5	148·8	10·1	63·6	7·9	3·7	6·29	14·72	97·4	0·91
49 W₃	155·9	127·8	16·6	52·3	21·1	2·8	3·15	7·70	95·0	0·98
49 W₄	156·9	116·3	22·7	42·6	23·8	3·7	1·88	5·13	92·5	0·94
36 W₅	169·7	122·7	12·2	41·5	26·1	1·4	3·38	10·08	91·2	0·89
48 W₆	78·6	114·2	34·1	50·7	11·3	—	1·49	3·35	84·5*	0·99
52 W₇†	164·1	104·0	44·6	47·4	12·6	4·3	1·06	2·33	88·0	1·03
52 W₈‡	163·9	106·1	45·9	47·0	15·0	4·1	1·02	2·33	91·2	1·02
11 W₉	133·0	114·8	54·0	51·5	11·8	8·9	0·95	2·13	100·6	1·00
11 W₁₀	133·0	117·8	55·4	51·2	8·1	—	0·92	2·13	91·3*	0·94

  * Non-reducing material was not analysed. C recovered would not be complete.
  † 0·8 m.mol. of lactic acid produced per 100 m.mol. of fermented glucose.
  ‡ 1·5 m.mol. of lactic acid produced per 100 m.mol. of fermented glucose.

the ratios of CO₂ to acetic acid from 0·92 to 6·29, whilst the succinic acid fluctu- ates from 7·9 m.mol. to 26·1 m.mol. per 100 m.mol. of fermented glucose. Virtanen and Van Niel found the ratios of CO₂ to acetic acid to be substantially 1. The ratio of propionic to acetic acid usually has been found to be approxi- mately 2 although Van Niel found ratios as high as 5. This increased yield of reduced products was explained by a donation of hydrogen by compounds occurring in the yeast-water medium. Such an explanation is not acceptable in the present case inasmuch as the redox indexes show that in the majority of fermentations there was no excess of reduced products.

Since these results differ from those found by other investigators the accuracy of the analyses might be questioned but the carbon balance, as well as the redox index, shows that the analyses are, in general, satisfactory. The purity of the cultures is hardly to be questioned. Represented among the cultures studied are those received from four different investigators. It is evident that the present schemes of the propionic acid fermentation are not entirely satisfactory, inasmuch as it is impossible to apply experimental values to the schemes. The problem of the mechanism of the fermentation therefore requires further investigation.

Schemes of glucose dissimilation generally represent the hexose chain as being split into two 3-carbon molecules, an exception being Virtanen's 4- and 2-carbon cleavage. The 3-carbon scheme requires that every 2-carbon product be accompanied by a 1-carbon compound. It is evident that the CO_2 is not equivalent to the acetic acid in a number of fermentations. The large excess of CO_2 suggests the occurrence of a 2-carbon intermediary which is synthesised into a compound of higher number of carbon atoms. Succinic acid is a product which might thus originate. Each molecule of succinic acid formed would result in two molecules of CO_2 or some other 1-carbon compound. In some fermentations the quantities of CO_2 found are greater and in others less than that required by this hypothesis ($CO_2 \equiv$ acetic $+ 2x$ succinic acid). The occurrence of CO_2 in quantities greater than can be accounted for by the formation of succinic acid through a synthesis indicates there may be another source of CO_2. The chemical structure of the non-reducing compound is not known, but there is apparently no correlation between it and the quantities of CO_2 produced. The suggestion is made that CO_2 originates in the formation of propionic acid. A decarboxylation of succinic acid would yield propionic acid and CO_2.

It is realised that these proposals as presented are somewhat speculative. Supporting evidence will be given later, but to complete a possible scheme of the fermentation the results must be accounted for in which the yields of CO_2 are less than the calculated amounts when succinic acid is assumed to be formed by a synthesis. Two possibilities are suggested. The first is that succinic acid is formed by a 4- and 2-carbon cleavage of the glucose molecule thus yielding succinic acid without directly involving a 1-carbon compound. The second is that the succinic acid is formed by a synthesis and that part of the CO_2 (1-carbon compound) is utilised subsequent to its formation. Wood and Werkman [1936] presented evidence that CO_2 is utilised by the propionic acid bacteria during the fermentation of glycerol and pointed out that the evidence for Virtanen's 4- and 2-carbon cleavage is not conclusive. Although proof of the second suggestion cannot be considered conclusive until direct evidence is obtained that the propionic acid bacteria utilise CO_2 during the fermentation of glucose, our present information suggests such an occurrence. Possibly both the 4- and 2-carbon cleavage and CO_2 utilisation occur. Further investigation of this problem is under way.

It is not our purpose to claim in the present paper that the occurrence of the suggested reactions has been proved but to show that the data are in agreement with such a scheme. It is apparent that numerous reactions may give the same relative quantities of CO_2, succinic acid etc., as those involved in the formation of succinic acid from acetic acid. For example, the condensation of two molecules of pyruvic acid to diketoadipic acid with a subsequent splitting off of two molecules of CO_2 yielding succinaldehyde and oxidation of the succinaldehyde to succinic acid yields the same quantitative results.

Apart from the data there is evidence substantiating the formation of succinic acid by synthesis as shown by Wood and Werkman [1936]. The future problem is one of determining the intermediate compounds of the condensation. Evidence

of the activation of acetic acid has been obtained by the authors and these results are discussed in relation to this problem in a separate paper [Stone *et al.*, 1936].

The formation of propionic acid from succinic acid finds support in the authors' [1935] investigations showing that succinic acid occurs as an intermediary and may be decomposed. Hitchner [1934] obtained evidence that succinic acid is dissimilated to volatile acids in the presence of a simultaneous fermentation of carbohydrates. Shaw and Sherman [1923] presented data showing that their culture produced propionic and acetic acids in a solution of peptone and succinate; other investigators have not confirmed this, however. That the propionic acid bacteria can dissimilate succinic acid under certain conditions is definitely established but the mechanism and products of the dissimilation have not been determined. The dissimilation is represented as a decarboxylation. It is evident that the succinic acid could be destroyed by other reactions which yield CO_2, thus giving the observed quantities of CO_2. If the reaction proceeds by the Thunberg series and pyruvic acid is formed it is probable that part of the pyruvic acid will be oxidised to acetic acid and CO_2. This oxidation would necessitate an increased reduction and thus an increase in propionic acid. The result would be an increase in CO_2 and propionic acid. For simplicity the change is shown as a decarboxylation in the following scheme (Fig. 1).
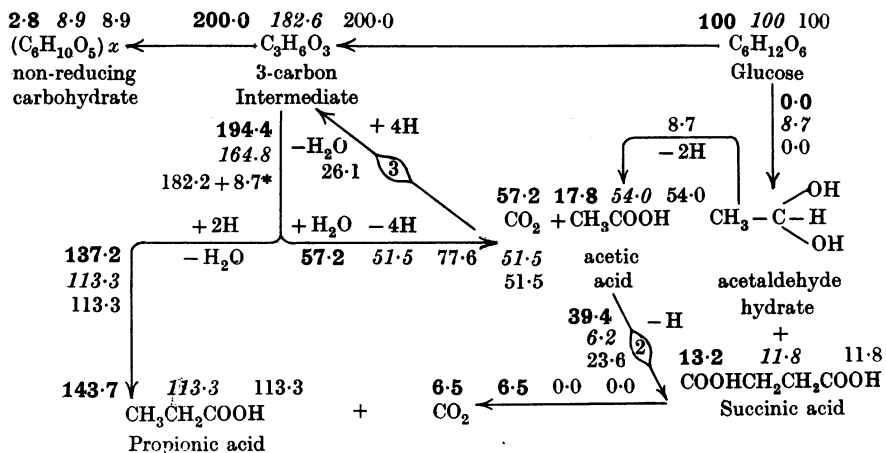

Fig. 1. Quantitative data and balances on substitution in the scheme of glucose dissimilation.
* 3-carbon compound from CO_2.

Our results (Fig. 1) illustrate the agreement between the data and schemes involving the foregoing suggestions. The quantities of CO_2, acetic acid and succinic acid have been used as a starting basis for substitutions. Fermentation $34W_1$, Table II, may be used as an example in which the glucose may have been dissimilated entirely by a 3-carbon cleavage. In this case the formation of 17·8 m.mol. of acetic acid and 13·2 m.mol. of succinic acid would give rise to 44·2 m.mol. of CO_2. 19·4 m.mol. of CO_2 are therefore unaccounted for. In the formation of propionic acid from succinic acid 3·0 m.mol. of CO_2 are formed for each m.mol. of propionic acid produced and 19·4/3 or 6·5 m.mol. of propionic acid would be produced from the succinic acid to give the observed value of CO_2. 39·4 m.mol. of acetic acid are required for the 13·2 m.mol. of succinic acid and 6·5 m.mol. of propionic acid. 39·4 + 17·8 = 57·2 m.mol. of 3-carbon intermediate which would be dissimilated to acetic acid. The m.mol. of 3-carbon compound

corrected for the non-reducing material *minus* 57·2 gives the quantity of propionic acid produced from the 3-carbon intermediate. This quantity of propionic acid (137·2) *plus* that formed from succinic acid gives the calculated propionic acid (143·7). The experimental value is 139·1. Since the carbon recovery is not quite complete the calculated and observed values cannot be in perfect agreement. The calculated values of $+H$ and $-H$ are 274·4 and 268·2 respectively.

Fermentation $11W_9$ may be cited as an example in which a 4- and 2-carbon cleavage may have occurred or a utilisation of CO_2. Consider first the 4- and 2-carbon cleavage (data in italics). If all of the acetic acid and succinic acid were produced following a 3-carbon cleavage of the glucose molecule there would be formed 77·6 m.mol. of CO_2. The observed value is 51·5. Each molecule of glucose fermented to succinic acid and acetic acid by the 4- and 2-carbon cleavage would decrease the quantity of CO_2 produced by 3 m.mol. 77·6−51·5 divided by 3 gives 8·7, the smallest quantity of glucose which could undergo a 4- and 2-carbon cleavage and give a calculated quantity of CO_2 equal to the observed. Using 8·7 as the glucose fermented by a 4- and 2-carbon cleavage, substitutions have been made as described above. The observed propionic acid is 114·8 and the calculated 113·3, $+H=226·6$, $-H=229·6$.

Calculations from the same fermentation are shown in which there is an assumed utilisation of CO_2. The method of calculation is evident. The observed propionic acid is 114·8 and the calculated 113·3, $+H=331·4$, $-H=334·0$.

It is evident that the scheme of fermentation meets the requirements of the quantitative data. Although a number of the fermentations do not give as good balances as the two chosen, they substantiate the scheme. The authors have purposely omitted showing intermediate reactions involved in the fermentation for the reason that present information does not warrant definite conclusions and because the intermediate reactions are not important to the calculations. No information is available as to the status of phosphoglyceric acid in this fermentation. The indicated synthesis of CO_2 to a 3-carbon intermediate is speculative.

At present it is impossible to explain accurately why results so markedly different from those of other investigators have been obtained in these fermentations. Culture 49W is a transplant of Van Niel's culture 4. Van Niel used an extract of 250 g. of yeast per litre of medium as a nitrogen source and his results indicate that a considerable quantity of fermentable material was included in the yeast extract. This is not the case with Bacto-yeast extract. After 30 days' fermentation at 30° with culture 34W in 0·4 % yeast extract and 1·4 % $CaCO_3$ there were produced 3·5 m.mol. of volatile acid, 4·7 m.mol. of CO_2, 0·3 m.mol. of succinic acid and no non-reducing sugar per litre of medium. Oxidation-reduction balances calculated from Van Niel's results are not satisfying and suggest that the nitrogen source materially affected the carbohydrate metabolism. It is difficult to speculate as to the mechanism of fermentation under such conditions. The use of various sources of nitrogen may have caused the different types of fermentation. The formation of succinic acid entirely from compounds of the yeast is excluded in our fermentation, for in exp. $36W_5$ 5·2 g. of succinic acid were produced per litre in a medium containing 4·0 g. of yeast extract.

SUMMARY.

The propionic acid fermentation of glucose has been investigated quantitatively and the results show certain points of disagreement with present schemes of the fermentation. The following conversions are suggested to be involved and the data are shown to fit schemes including these reactions.

1. The formation of succinic acid by a synthesis, possibly by the condensation of two molecules of acetic acid.

2. The intermediate dissimilation of succinic acid causing an increased production of propionic acid and CO_2.

3. A condensation of CO_2 and a subsequent utilisation of the synthesised product. The possibility is left that the 4- and 2-carbon cleavage occurs as suggested by Virtanen.

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