21. THE RELATIONSHIP OF BACTERIAL UTILIZATION OF CO_2 TO SUCCINIC ACID FORMATION[1]

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PREVIOUS investigations by Wood & Werkman [1938; 1940] have shown that CO_2 is utilized by the propionic acid bacteria in the dissimilation of a variety of substrates and that this fixation of CO_2 is inhibited by NaF. The utilization of CO_2 in the fermentation of glycerol is accompanied by an equimolar formation of succinic acid. It has therefore been proposed that in this fermentation succinic acid formation involves the combination of CO_2 and a 3-carbon compound. The discovery that NaF inhibits CO_2 utilization provided a tool for determining the validity of the proposal.

The present report considers the influence of conditions on the utilization of CO_2 and the formation of succinic acid in the dissimilation of glycerol and glucose by the propionic acid bacteria. Especially the influence of NaF is discussed. The results show that in the dissimilation of either glucose or glycerol, inhibition of CO_2 utilization causes an approximately equimolar reduction in the formation of succinic acid.

Methods

It was necessary to determine quantitatively in a rather large number of experiments the products formed by cell suspensions acting under an atmosphere of CO_2; therefore a small volume of reaction mixture was used to reduce the required amount of cell suspension. Also the determination of CO_2 in a dissimilation under an atmosphere of CO_2 requires that changes in the volume (or pressure) of CO_2 gas above the fermentation liquor must be measured. Therefore the following technique was developed for determination of the products.

The dissimilation was carried out in a 125 ml. Erlenmeyer flask with two side cups and a ground glass joint fitted to a Barcroft-Warburg manometer having a two-way stopcock (Fig. 1). The flask was immersed in a water bath at 30° and was oscillated continuously by the usual shaking device 100 times per min. through an amplitude of 5 cm. A small mercury trap and Liebig bulb containing 5 ml. of 4*N* carbonate-free NaOH was attached to the stopcock as shown. The alkali was protected by a small soda lime tube.

The experiments were set up as follows: 24 ml. of substrate, buffer etc. were pipetted into the main chamber of the reaction flask and 4 ml. of the suspension of bacteria and 2 ml. of 10*N* H_2SO_4 into the two side cups. The flask was then attached to the manometer with rubber bands and a small piece of wood was inserted in the ground glass joint so that CO_2 could pass through. A vigorous current of CO_2 saturated with water was then directed through the stopcock into the flask for 15 min. and finally, for a few minutes through the mercury trap

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to remove the air from the system. It was necessary to shake the apparatus frequently during the flushing with CO_2 in order to saturate the solutions. The apparatus was next placed in the water bath. The reading of the manometer was taken after equilibrium was established, the bacteria were tipped into the main chamber, the alkali bulb and soda lime tubes were attached and the stopcock opened to the mercury trap allowing passage of CO_2 into the alkali. At the conclusion of the experiment the 2 ml. of acid in the side cup were poured into the main chamber and after equilibrium was established the manometer was read. The difference in the initial and final readings, after correction for barometric change, multiplied by the flask-constant, equals the change in volume of the CO_2. The CO_2 in the alkali was determined as follows: the alkali was washed from the Liebig bulb, acidified with H_2SO_4, and the liberated CO_2 was absorbed in a weighed potash bulb. The CO_2 determined manometrically was added to the CO_2 of the alkali. A similar determination of CO_2 was made on a mixture to which no bacteria were added. By difference the CO_2 produced or utilized during the dissimilation was calculated.

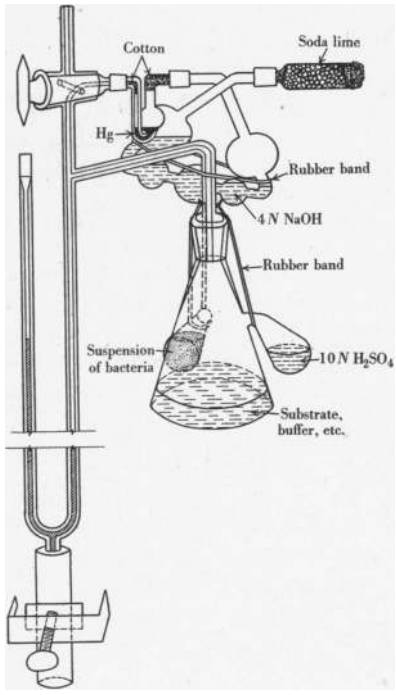

Fig. 1. Manometric apparatus.

The 28 ml. of fermented mixture plus the 2 ml. of H_2SO_4 were treated as follows.

Two ml. of the mixture were hydrolysed for 2·5 hr. in 25 ml. of boiling 3N HCl and total reducing sugars were then determined according to Munson & Walker [1906] and calculated as glucose. Reducing sugars of an unhydrolysed sample were also determined. An equivalent quantity of the original cell suspension was hydrolysed for determination of its content of non-reducing sugar. The non-reducing sugar of the original cells plus that in the unhydrolysed reaction mixture was subtracted from the total sugar of the hydrolysed reaction mixture to obtain the non-reducing sugar formed by the dissimilation.

The remainder of the fermented mixture was centrifuged to remove cells. One ml. was diluted to 25 ml. and a suitable aliquot used for determination of the unfermented substrate. The glucose was determined by the method of Stiles *et al.* [1926], and glycerol as described by Wood & Werkman [1940].

Propyl alcohol is formed in the fermentation of glycerol. The alcohol was distilled from 14 ml. of the reaction mixture which was made alkaline to phenolphthalein with 10N NaOH. 10 ml. of distillate were collected and oxidized with dichromate. The resulting acid was recovered by steam distillation and determined by titration (unpublished method).

The volatile acids were recovered by steam distillation from the acidified residue of the alcohol distillation in the case of glycerol fermentations. In the case of glucose fermentations 10 or 14 ml. of the centrifuged mixture were used and distilled to about 6 ml. in a micro-Kjeldahl flask. Then an additional 14 volumes (84 ml.) were collected by steam distillation. The distillate was made up to

110 ml. and the volatile acids were determined by the partition method [Osburn *et al.* 1933; 1938]. Pyruvic acid, when present, was estimated in the volatile acid distillate by the iodoform reaction [Goodwin, 1920] and a correction was made for it in the volatile acid determination. About 65 % of the pyruvic acid is distilled. HF (from NaF) also interferes; about 65 % volatilizes during steam distillation. This acid was precipitated as the Ca salt as follows. The distillate was made alkaline with $Ca(OH)_2$ and evaporated to about 2·5 ml. in a 50 ml. Erlenmeyer flask. The liquid was then filtered through a small Gooch crucible and the flask and crucible were washed with small portions of water, about 6 ml. in all. The filtrate and washings were collected in a micro-Kjeldahl flask and volatile acids were recovered from the acidified solution by steam distillation.

0·2 ml. of the reaction mixture was used for a qualitative test for pyruvic acid by the sodium nitroprusside reaction. When pyruvic acid was present 14 ml. of the reaction mixture were extracted continuously with ether for 24 hr. 10 ml. of water were added to the extract and the ether was distilled off. The solution was then made up to 50 ml. 25 ml. were used for determination of pyruvic acid and the remaining 25 ml. were treated as follows. The volatile acids were removed by steam distillation and the total non-volatile acid in the residue of distillation titrated. The succinic acid was precipitated from this solution as the Ag salt and determined by weight. Lactic acid was estimated by the method of Friedemann & Graeser [1933] on the filtrate from the succinic acid determination with the exception of those experiments in which the titration value indicated that there was no lactic acid; i.e. when the total acid was equivalent to that of the succinic and 35 % of the pyruvic and hydrofluoric acids. Pyruvic acid was never formed in the fermentation of glycerol; in this case 10 ml. of reaction mixture were extracted with ether for the succinic acid determination.

The original reaction mixture contained the following concentrations of constituents: substrate 1·60 %, $NaHCO_3$ 1·4 %, cell suspension 0·05 g. wet bacteria per ml. of reaction mixture and NaF as indicated. When phosphate buffer (0·1 *M*) was used, it was in addition to the $NaHCO_3$ and was in a molar ratio of 79 of KH_2PO_4 to 21 of K_2HPO_4. The original concentration of substrate was made up accurately by weight. The time of incubation was usually 30 hr. for glucose and 50 hr. for glycerol. No correction was made for endogenous dissimilation other than for the non-reducing sugar in the cells. On the basis of glucose this value ranged from 0·16 mg. to 1·80 mg. per ml. of reaction mixture for the different cells used and was usually about 0·30 mg.

The cells used in the reaction mixture, *Propionibacterium pentosaceum* 49 W, were harvested after 5 days' growth at 30° in medium containing glucose or glycerol 0·5 %, $NaHCO_3$ 1·0 % or phosphate buffer (0·1 *M*, *p*H 6·9) and Difco yeast extract 0·4 %. The cells were washed twice with water before use.

Results shown in Table 4 were obtained with proliferating cells. The medium contained glycerol or glucose 2·0 %, $NaHCO_3$ 1·75 %, Difco yeast extract 0·4 % and NaF when indicated 0·02 *M*. In the glucose experiments (no. II in the Tables) 0·1 *M* phosphate buffer *p*H 6·15 was used in addition to $NaHCO_3$. Incubation was at 30° for 20 days in the case of glucose and 30 days in that of glycerol. The culture was 49 W (*P. pentosaceum*). Procedures and methods of analysis were those described by Wood & Werkman [1938].

Experimental results

The experimental results are presented in m*M* per 100 m*M* of substrate fermented. The carbon balance and redox index (perfect index = 1·00) are for the most part satisfactory, except in those experiments where there was apparently

Table 1. *Influence of NaF on formation of succinic acid and utilization of CO_2 by cell suspension of 49 W*

Cells were grown in glycerol, yeast extract, phosphate medium

Exp. no.	Conc. of NaF in reaction mixture *M*	Phosphate in reaction mixture	Substrate	Substrate fermented per l. m*M*	Products per 100 m*M* substrate: Propionic acid m*M*	Acetic acid m*M*	CO_2 m*M*	Succinic acid m*M*	Propyl alcohol m*M*	Pyruvic acid m*M*	Non-reducing material m*M*	Carbon recovery %	O/R
II	0·000	Yes	Glycerol	161·3	46·8	3·80	−38·7	39·1	4·42	0·0	2·55	98·4	1·01
	0·0125	Yes	,,	23·8	98·6	5·52	−3·48	3·90	3·14	0·0	0·00	109·3	0·91
III	0·000	Yes	,,	158·4	60·4	4·68	−28·5	27·5	3·97	0·0	3·05	100·6	0·99
	0·015	Yes	,,	46·9	86·3	9·59	−6·67	4·25	2·75	0·0	4·60	107·6	0·97
	0·000	—	,,	159·4	65·5	4·48	−25·5	23·2	3·15	0·0	1·15	96·7	0·98
IV	0·000	—	,,	172·2	66·3	5·14	−39·0	36·7	0·00	0·0	0·0	104·7	0·95
	0·015	—	,,	108·0	97·1	4·05	−11·8	6·21	0·00	0·0	0·0	103·9	0·88
I	0·000	—	Glucose	82·6	78·7	26·6	22·0	32·3	—	—	17·9	91·3	0·97
	0·015	—	,,	64·5	130·3	13·4	54·4	14·0	—	—	5·21	93·2	0·94
II	0·000	Yes	,,	83·0	80·9	14·8	15·6	42·9	—	—	20·8	97·3	0·92
	0·015	Yes	,,	26·5	—	—	—	0·23	—	—	—	—	—
III	0·000	Yes	,,	83·0	93·0	17·1	26·1	28·7	—	4·95	17·4	95·6	0·92
	0·010	Yes	,,	69·3	120·6	17·5	51·1	13·2	—	4·73	10·7	96·6	1·00

Table 2. *Formation of succinic acid by bacteria grown in different types of media and the effect of NaF on the fermentation*

Exp. no.	Medium used for growth of cells	Conc. of NaF in reaction mixture *M*	Phosphate in reaction mixture	Substrate	Substrate fermented per l. m*M*	Products per 100 m*M* of substrate: Propionic acid m*M*	Acetic acid m*M*	CO_2 m*M*	Succinic acid m*M*	Propyl alcohol m*M*	Non-reducing material m*M*	Carbon recovery %	O/R
I	Glycerol, $NaHCO_3$, yeast extract	0·000	—	Glucose	83·0	104·8	26·7	55·6	6·87	—	10·8	85·4	1·12
		0·015	—	,,	69·8	126·6	28·6	61·8	5·85	—	6·22	93·2	1·02
II	Glucose, $NaHCO_3$, yeast extract	0·000	—	,,	83·0	101·8	27·9	49·3	7·09	—	31·2	104·3	1·04
		0·015	—	,,	83·0	118·6	27·2	61·3	7·18	—	2·68	88·2	1·09
		0·000	—	,,	109·3	74·9	5·38	−0·90	8·39	—	—	—	—
III	Glucose, phosphate, yeast extract	0·000	Yes	,,	48·0	—	—	44·7	0·0	—	17·9	—	—
		0·000	Yes	Glycerol	37·5	—	—	−6·24	4·83	—	0·0	—	—
IV	Glucose, $NaHCO_3$, yeast extract, NaF 0·02 *M*	0·000	Yes	Glucose	83·0	105·9	11·8	44·9	14·5	—	22·0	93·1	0·99
		0·020	Yes	,,	83·0	116·4	12·9	51·3	14·3	—	13·9	94·5	1·00
		0·000	Yes	Glycerol	157·5	73·9	6·25	−6·46	7·67	7·15	0·77	95·1	0·99
		0·020	Yes	,,	41·8	91·3	5·11	−3·28	4·47	4·14	0·0	103·7	0·95
		0·000	—	,,	157·4	73·4	4·84	−9·27	5·44	6·75	3·32	94·2	0·94
V	Glycerol, phosphate, yeast extract, NaF 0·02 *M*	0·000	Yes	Glucose	28·4	—	—	—	4·59	—	—	—	—
		0·020	Yes	Glucose	23·9	—	—	—	3·77	—	—	—	—

Table 3. *Influence of phosphate on formation of succinic acid by cell suspensions*

Exp. no.	PO_4 in reaction mixture	Substrate	Substrate fermented per l. m*M*	Products per 100 m*M* substrate: Propionic acid m*M*	Acetic acid m*M*	CO_2 m*M*	Succinic acid m*M*	Propyl alcohol m*M*	Pyruvic acid m*M*	Lactic acid m*M*	Non-reducing material m*M*	Carbon recovery %	O/R
I	No	Glucose	83·0	100·9	33·3	51·5	4·80	—	—	—	30·5	104·2	1·07
	Yes	,,	83·0	92·8	29·6	44·2	14·2	—	—	—	23·5	96·6	1·10
II	No	,,	83·0	102·0	18·3	43·3	1·19	—	10·5	26·4	9·72	93·2	0·96
	K_2HPO_4	,,	83·0	89·2	9·37	34·3*	6·90	—	13·7	16·2	20·5	93·6*	—
	Yes	,,	83·0	90·3	11·21	38·4	9·75	—	13·5	14·3	19·8	95·4	1·10
	No	Glycerol	158·6	82·0	5·51	−6·25	6·25	4·40	0·0	0·0	0·0	96·2	0·98
	Yes	,,	156·5	76·0	4·68	−9·61	13·0	5·82	0·0	0·0	1·28	101·6	1·00

* CO_2 not determined; value given was calculated on basis of O/R balance. Carbon recovery was calculated using the estimated CO_2 value.
I. Cells were grown in glycerol, yeast extract, $NaHCO_3$.
II. Cells were grown in glucose, yeast extract, $NaHCO_3$.

Table 4. *Influence of NaF on formation of succinic acid and utilization of CO_2 in the dissimilation of glucose*

Exp. no.	Conc. of NaF in reaction mixture *M*	Propionic acid m*M*	Difference in propionic acid m*M*	Acetic acid m*M*	CO_2 m*M*	Difference in CO_2 m*M*	Succinic acid m*M*	Difference in succinic acid m*M*	Pyruvic acid m*M*
I	0·000	94·8	41·6	32·4	26·8	30·6	39·3	−24·6	
	0·015	137·4		14·1	57·4		14·7		
II	0·000	112·6	22·5	20·7	31·6	25·6	34·8	−20·0	6·0
	0·010	135·1		19·7	57·2		14·8		5·3

Table 5. *Influence of NaF on formation of succinic acid and utilization of CO_2 by proliferating cells (49 W)*

Exp. no.	Conc. of NaF in medium *M*	Substrate	Substrate fermented per l. m*M*	Products per 100 m*M* substrate: Propionic acid m*M*	Acetic acid m*M*	CO_2 m*M*	Succinic acid m*M*	Propyl alcohol m*M*	Pyruvic acid m*M*	Lactic acid m*M*	Non-reducing material m*M*	Carbon recovery %	O/R
I	0·020	Glycerol	140·2	83·2	4·19	−5·88	5·26	1·29	0·53	0·0	0·21	93·4	1·07
	0·000	,,	212·2	67·9	2·24	−23·3	23·5	1·95	0·0	0·0	1·77	98·5	1·03
I	0·020	Glucose	103·3	116·1	13·6	51·9	7·26	—	3·15	16·1	4·95	90·7	0·98
	0·000	,,	106·3	113·9	28·0	52·1	11·3	—	1·06	3·01	6·90	91·4	1·02
II	0·020	,,	94·9	105·0	17·4	52·7	10·7	—	0·0	38·8	1·17	94·8	1·11
	0·000	,,	108·3	124·5	21·1	57·7	16·0	—	0·0	1·99	4·40	95·0	1·05

an error or all the products were not determined. Lactic and pyruvic acids were not determined in some of the earlier experiments. These products are sometimes formed, particularly from glucose; a more accurate balance might have been obtained had they been estimated.

Discussion

It is evident from a consideration of Tables 1 and 2 that the manner in which cells are grown influences their ability, as a cell suspension, to form succinic acid from glycerol or glucose. Higher yields of succinic acid were consistently obtained from cells which had been grown on the glycerol-phosphate-yeast extract medium (Table 1) than from cells grown on other types of media (cf. column 2, Table 2). The largest yield in Table 2 was 14·5 mM of succinic acid as compared with values ranging from 27·5 mM to 42·9 mM in Table 1; this, in spite of the fact that while growing on a glycerol-phosphate-yeast extract medium, the organisms produce less succinic acid than when growing on certain of the other media (e.g. glycerol-$NaHCO_3$-yeast extract). Thus the results can hardly be attributed to adaptive enzymes. It would seem that the ability of cell suspensions to form succinic acid depends upon other factors.

It appeared possible that lack of phosphate might be a limiting factor when cells were used that had been grown in a medium with no added phosphate. The results (Table 3) indicate that this explanation may in part be valid. Cells were grown in a medium to which no inorganic phosphate was added and were tested with and without the addition of phosphate. Both with glucose and glycerol the addition of phosphate increased the yield of succinic acid. In exp. I of Table 3, 4·8 mM of succinic acid were formed when phosphate was not added, and 14·2 mM when it was added. The other experiments show the same trend though the variation is not as large. The highest yield of succinic acid in these experiments, however, was by no means equivalent to that obtained with cells grown on glycerol-phosphate-yeast extract medium (Table 1). Apparently factors in addition to phosphates are involved which are inherent in the cells and determined during growth. Growth in a medium containing phosphate is not sufficient in itself to cause development of active cells since those grown in glucose-phosphate-yeast extract medium did not form much succinic acid (Table 2, exp. III). Phosphate need not be added to obtain a substantial yield of succinic acid when the cells used are from glycerol-phosphate-yeast extract medium (Table 1, exps. 1 and IV). In all probability sufficient phosphate is introduced with the cells to supply their requirements. The results in Table 3 show clearly that phosphate stimulates the formation of succinic acid even though large yields are not obtained. The influence of phosphate is not due to change in pH since the dibasic salt had the same effect as the more acid mixture.

The effect of cells grown in a medium containing 0·02 M NaF was determined inasmuch as Wiggert & Werkman [1939] have shown that CO_2 production from glucose by suspensions of such cells is increased in the presence of NaF. It seemed probable that the increase was caused by inhibition of the utilization of CO_2. The possibility of using cells insensitive to NaF [Wiggert & Werkman, 1939] for study of CO_2 utilization was attractive, for it is difficult when using fluoride-sensitive cells to select a concentration of fluoride such that the utilization of CO_2 is inhibited and dissimilation is not. The results obtained with cells grown in NaF (Table 2, exps. IV and V) indicate that they neither utilize much CO_2 nor produce large yields of succinic acid. The increased production of CO_2 from glucose in the presence of NaF is probably caused by suppression of the formation of non-reducing sugar. Thus more of the carbon of glucose is diverted to a gas-forming

mechanism. In all experiments of Tables 1 and 2 less non-reducing sugar was formed from glucose in the presence of NaF. There are, then, at least two ways by which NaF increases formation of CO_2; one, by inhibiting the formation of non-reducing sugars, and another, by inhibiting utilization of CO_2.

The influence of NaF on the dissimilation of glucose and glycerol, in which there was substantial formation of succinic acid and utilization of CO_2, is shown in Table 1. NaF inhibits the formation of succinic acid from both glucose and glycerol and the fixation of CO_2 in the dissimilation of glycerol. The decrease in fixation of CO_2 is accompanied by an almost identical decrease in succinic acid formation. For example, in exp. II, 38·7 mM of CO_2 were fixed and 39·1 mM of succinic acid formed in the absence of fluoride, whereas in the presence of fluoride the quantities were 3·48 and 3·90. This relationship is further evidence that the formation of succinic acid involves the utilization of CO_2. The quantitative relationships of the products formed by cell suspensions from glycerol are substantially those obtained with proliferating cells [Wood & Werkman, 1938]. Whenever the utilization of CO_2 increases, the yield of propionic acid decreases and of succinic acid increases. The amounts of acetic acid, propyl alcohol and non-reducing sugar are small and not significantly affected by the NaF or change in utilization of CO_2. Whether the decrease in propionic acid has any significance with regard to mechanism of utilization of CO_2 or is the result of diversion of the carbon to another compound cannot be answered at present. It has been established, however, that cell suspensions in the presence of propionic acid and CO_2 do not take up CO_2, nor does the addition of propionic acid to other substrates increase the utilization of CO_2.

Although the dissimilation of glucose is more difficult to explain than that of glycerol, there is no doubt that fluoride inhibits the formation of succinic acid and at the same time increases the production of CO_2. In exp. I, Table 1, fluoride addition resulted in an increased formation of CO_2 from 22·0 to 54·0 mM of CO_2 and a decrease in succinic acid from 32·3 to 14·0 mM. These changes are probably due to the inhibition of the uptake of CO_2.

It has been shown that fluoride causes an increase in the formation of CO_2 (on the basis of glucose used) by decreasing the amount of non-reducing sugar formed. Since the non-reducing sugar is assumed to be a polymer of glucose, the results can also be calculated in such a manner that the non-reducing sugar is considered as unfermented substrate. Variations due to non-reducing sugar are thus removed and the relationship of CO_2 and succinic acid is more clearly apparent (Table 4). It is evident that the main changes caused by NaF are an increase of propionic acid and CO_2 and a decrease of succinic acid. Moreover, the increased formation of succinic acid in the absence of NaF is accompanied by an almost equivalent decrease in yield of CO_2 (30·6 and −24·6 in one case and 25·6 and −20·0 in the other). This is strong evidence that CO_2 is utilized in the fermentation of glucose with formation of succinic acid as occurs in the glycerol fermentation. However, from certain experimental evidence it seems reasonable to suppose that the formation of succinic acid through utilization of CO_2 is not the only mechanism of its formation. In Table 4 if all the succinic acid had been formed by means of utilization of CO_2 there would have been produced at least 65–70 mM of CO_2 (observed CO_2 plus succinic acid), with only 14–32 mM of acetic acid. It does not seem probable that this occurred for it is likely that the 2- and 1-carbon compounds are formed initially in equimolar quantities. It is more likely that succinic acid is formed both by utilization of CO_2 and by condensation of acetic or pyruvic acid. It is noteworthy in this respect that cells obtained by growth in a NaF-glycerol-phosphate-yeast extract medium do not have a marked ability

to form succinic acid or to utilize CO_2 actively (Table 2, exp. V). Apparently the fluoride-sensitive mechanism of succinic acid formation was not developed in cells grown in the presence of NaF or in the other types of media considered in Table 2. In general the dissimilation of glucose is less sensitive to NaF than is the glycerol dissimilation and likewise the formation of succinic acid from glucose is inhibited less.

From a consideration of experimental evidence, it seems probable that formation of succinic acid by fixation of CO_2 involves the Embden-Meyerhof-Parnas mechanism of glucose or glycerol dissimilation. On the other hand, the formation of succinic acid by condensation of acetic or pyruvic acid may occur independently of the Embden-Meyerhof-Parnas reaction; i.e. by a mechanism of glucose or glycerol dissimilation which is insensitive to fluoride. Werkman *et al.* [1937] and Wiggert & Werkman [1939] have offered convincing evidence that there are at least two mechanisms of glucose dissimilation possessed by propionic acid bacteria: one sensitive to fluoride and proceeding according to the Embden-Meyerhof-Parnas scheme; the other insensitive to fluoride. It has been shown [Wood & Werkman, 1940] that phosphoglyceric acid is not dissimilated in a concentration of fluoride which inhibits utilization of CO_2. Therefore any succinic acid arising in the presence of fluoride cannot have been formed through the Embden-Meyerhof-Parnas reactions or by fixation of CO_2. Moreover, cells not possessing the mechanism for breakdown of phosphoglyceric acid but possessing the fluoride-insensitive mechanism of dissimilation (cells grown in medium containing NaF) do not fix a significant quantity of CO_2 even in the absence of NaF (Table 2, exps. IV and V). The implication is that the absence of the Embden-Meyerhof-Parnas mechanism precludes utilization of CO_2. Furthermore, phosphate slightly stimulates utilization of CO_2 (Table 3). All of these facts indicate that fixation of CO_2 involves a phosphorylating mechanism.

A few experiments were conducted to determine the effect of fluoride on dissimilations by proliferating cells. In the case of the fermentation of glycerol (Table 5, exp. I), fluoride inhibited fixation of CO_2 and formation of succinic acid as with the cell suspension. The dissimilation of glucose does not show such definite effects, possibly because of the small amount of CO_2 fixed by the proliferating cells. The formation of succinic acid was also largely uninhibited. Apparently under the conditions of these experiments glucose was fermented with only a small fixation of CO_2 and the greater part of the succinic acid was formed by acetic or pyruvic acid condensation. In the presence of fluoride considerable lactic acid was formed from glucose. This formation of lactic acid reduces the production of CO_2, whereas an increase might have been expected owing to the inhibition of fixation of CO_2 by fluoride. That there was some fixation of CO_2 is indicated by the observed small decrease in succinic acid formation in the presence of fluoride.

An understanding of the mechanism of fixation of CO_2 may offer new avenues of investigation into the chemistry of photosynthesis [cf. Gaffron,[1] 1939]. Obviously the same type of mechanism could be common to the two processes. Preliminary results have shown that NaF affects the fermentation of galactose by *E. coli* much as it does the propionic acid fermentation; i.e. the production of CO_2 is increased and the succinic acid is decreased when fluoride is added. These results are interpreted as evidence of utilization of CO_2 with formation of succinic acid by colon bacteria. Fixation of CO_2 with formation of succinic acid

[1] Gaffron [1939] apparently misinterpreted the results of our previous investigations for he states that a net decrease in CO_2 has not been shown in the propionic acid fermentation.

may be a general biological reaction. Certainly activation of CO_2 by heterotrophic bacteria is a general phenomenon [cf. Wieringa, 1936; Woods, 1936; Barker, 1936; 1937 and Hess, 1938]. It should be emphasized, however, that there is one essential difference between the reactions referred to which involve fixation of CO_2. In the first place, there is the fixation as noted by the authors and Wieringa which involves reduction with a carbon-to-carbon linkage and, in the second place, that involving reduction with no linkage of carbon atoms. It is from an understanding of the carbon-to-carbon linkage that an elucidation of the reaction of assimilation may be expected.

Previously the authors have suggested that synthesis of citric acid by moulds may involve fixation of CO_2. In this connexion it is interesting that Johnson *et al.* [1939] have found that 0·01 *M* NaF inhibits formation of citric acid by *Aspergillus niger*. They suggest that inactivation of succinic dehydrogenase by NaF is the true cause of the inhibition. According to their proposals citric acid may be formed by a 4- and 2-carbon splitting of glucose or a breakdown to three 2-carbon compounds followed by synthesis with formation of succinic acid. The succinic acid is in turn activated by succinic dehydrogenase to combine with acetic acid to yield citric acid. In view of the results reported here, the NaF may be inhibiting a synthesis involving CO_2, and thus be preventing citric acid formation.[1] There is in reality no substantial proof of the often proposed 4- and 2-carbon cleavage of glucose [cf. Wood & Werkman, 1938].

Summary

The activity of cell suspensions of *Propionibacterium pentosaceum* (49 W) in fixing CO_2 depends on the medium used for growth. A glycerol-phosphate-yeast extract medium yields active cells. Inhibition of utilization of CO_2 by NaF causes a simultaneous decrease in the formation of succinic acid. This is further evidence that succinic acid may be formed by union of 1- and 3-carbon compounds. Apparently there is a second mechanism for the formation of succinic acid (particularly in glucose dissimilation) which is insensitive to fluoride. This fluoride-insensitive formation of succinic acid probably occurs by the condensation of acetic or pyruvic acid. CO_2 utilization is believed to be associated with a fluoride-sensitive phosphorylating mechanism. Attention is drawn to the possibility that fixation of CO_2 with formation of succinic acid may be a general biological phenomenon.

[1] An explanation of the inhibitive action of NaF on the utilization of CO_2 by propionic acid bacteria based on inactivation of succinic dehydrogenase is hardly acceptable because malonate and pyrophosphate do not affect fixation of CO_2 [cf. Wood & Werkman, 1940].

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