2. THE FIXATION OF CO_2 BY CELL SUSPENSIONS OF *PROPIONIBACTERIUM PENTOSACEUM*[1]

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THE fixation of CO_2 by heterotrophic non-photosynthetic bacteria was first reported by Wood & Werkman [1936; 1938] in the case of the fermentation of glycerol by the propionic acid bacteria. It was established that the CO_2 utilized and the succinic acid formed are approximately equimolar. These results have been confirmed by Phelps *et al.* [1939]. Wood & Werkman [1938] have suggested that succinic acid is formed in this fermentation by synthesis from a 3-carbon compound and CO_2. The present investigation is a continuation of these studies to determine whether similar considerations apply to the fermentation of other substrates. It is not improbable that the formation of succinic acid by synthesis from CO_2 and a 3-carbon compound may occur more generally than has been suggested.

METHODS

Barcroft-Warburg manometric technique was used according to Dixon [1934]. 2 ml. of mixture were used in each cup unless otherwise stated. The temperature of the bath was 30°. The flasks were oscillated continuously 100 times per min. through an amplitude of 5 cm. *Propionibacterium pentosaceum* (49 W) was grown at 30° for 5 days in a medium of yeast extract (Difco) 0·4 %, glycerol 0·5 %, and 0·05 *M* phosphate buffer (*p*H 6·9). The growth was harvested by centrifuging and washed twice with distilled water by suspending 1 g. of wet bacteria in 20 ml. of water. Unless otherwise stated the reaction mixture contained the following concentrations of constituents: substrate 0·2 %, phosphate buffer 0·1 *M* (*p*H 6·15), and 0·025 g. wet bacterial paste per ml. The bacteria, phosphate buffer and other constituents (cf. Tables) were placed in the main chamber of the respirometer flask and the substrate in the side cup. Time of reaction was 18 hr. with the exception of the experiment described in Table 5. The atmosphere was CO_2 with one exception (cf. Table 2).

Under an atmosphere of CO_2 the phosphate buffer takes up much CO_2. During the dissimilation, CO_2 is liberated from the buffer by the acids which are formed. It was therefore necessary to determine the CO_2 bound by the buffer in order to calculate the CO_2 formed or utilized in the dissimilation. This CO_2 was determined manometrically in a mixture containing all constituents except the bacteria, by acidifying with 0·25 ml. of 3 *N* H_2SO_4. Likewise the CO_2 was liberated from the reaction mixture at the conclusion of the dissimilation. The CO_2 produced or utilized in the dissimilation of the substrate is the sum of the CO_2 liberated in the dissimilation and that obtained by acidification at the conclusion of the dissimilation minus the CO_2 originally bound by the buffer.

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The CO_2 formed by the bacteria in the absence of added substrate was usually measured (endogenous CO_2). This value was subtracted from that obtained when substrate was added in all experiments except those of Table 5. Although it is not certain that the endogenous dissimilation proceeds unchanged in the presence of added substrate, nevertheless, endogenous values were subtracted in experiments involving low substrate concentrations (0·2 %) because the gas volumes are small and failure to apply this correction might lead to erroneous conclusions with certain substrates. Since the cells in the absence of added substrate took up CO_2, subtraction of these values makes the apparent CO_2 uptake in the presence of substrate appear smaller; therefore any error does not yield evidence supporting CO_2 utilization.

Certain experiments (Table 2) were conducted in an atmosphere of N_2. In this case the CO_2 was absorbed in 0·3 ml. of $2N$ carbonate-free NaOH placed in one of the side cups of the flask. At the conclusion of the experiment the alkali was tipped into the main chamber and acidified with 0·4 ml. of $3N$ H_2SO_4. The liberated CO_2 was measured manometrically.

The procedure in the experiments of Table 5 required modification of technique in that the $NaHCO_3$ buffer contained more bound CO_2 than could be determined at one reading of the manometer. This difficulty was partly overcome by reducing the volume of the reaction mixture to 1 ml. (equivalent to approximately 3600 μl. CO_2 when 1·4 % $NaHCO_2$ was used). During the dissimilation a large part of this CO_2 was liberated so that the remaining bound CO_2 was within the limits of manometric determination.

The original bound CO_2 was determined by acidifying 0·6 ml. (without bacteria) of the 0·72 % $NaHCO_3$ mixture and 0·3 ml. of the 1·4 % $NaHCO_3$ mixture; each was equivalent to approximately 1000 μl. of CO_2. When the manometer was set initially at scale-readings 300 on the closed arm and 0 on the open arm, the 1000 μl. of gas displaced the manometer to 0 on the closed arm and approximately 300 on the open arm, final readings. These readings were adjusted to correspond to those that would be obtained if the measurements had been made at constant volume, using 150 as the zero point, by the following manipulations. The stopcock was opened and the Brodie solution adjusted to 300 in both arms of the manometer. Then the change in reading was determined when the manometer fluid was lowered to 150 in the closed arm. This value was added to the original reading (0) giving the value corresponding to a 150 setting. The correction for the final reading likewise was obtained by setting the manometer fluid at 0 and 0 and determining the change on adjustment to 150. This value was added to the final reading. Calculation has shown that there is a small error in this procedure but the errors of the two operations largely compensate each other.

The concentration of substrate in the original mixture was determined by weight. When reducing sugars were used, the residual sugar was determined by the method of Stiles *et al.* [1926]. Residual polyhydric alcohols were determined by oxidation with HIO_4. The polyhydric alcohol (less than 6 mg. in 10 ml.) plus 2 ml. of $3{\cdot}6N$ H_2SO_4 and 25 ml. of $0{\cdot}0625N$ HIO_4 was heated for 10 min. on a steam bath. The mixture was cooled and 2 ml. of 50 % KI were added. After 3 min. 25 ml. of water were added and then by pipette 25 ml. of $0{\cdot}5N$ $Na_2S_2O_3$ with vigorous shaking. The titration was completed with $0{\cdot}01N$ $Na_2S_2O_3$.

(Blank titration − titration of determination) × normality of $Na_2S_2O_3$ × 23·00 = mg. of glycerol. For erythritol the ml. of N $Na_2S_2O_3$ are multiplied by 20·33, for adonitol by 19·00 and for mannitol by 18·20.

EXPERIMENTAL

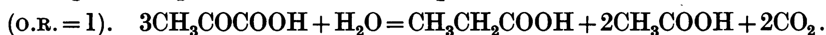
Comparative fixation of CO_2 with various substrates. Glycerol was the only substrate which had been shown by previous investigations to be dissimilated with an uptake of CO_2. Moreover, the action of cell suspensions had not been investigated. Dissimilation of a number of substrates by cell suspensions was therefore undertaken. Table 1 gives representative results from one of a number

Table 1. *Comparative utilization of CO_2 by cell suspensions of* P. pentosaceum *(49 W) with various substrates*

Bacteria with no substrate took up 64 μl. of CO_2

Substrate	Pyruvic acid	Lactic acid	Glyceraldehyde	Glucose	Dihydroxyacetone	Arabinose	
μl. CO_2 per 2 ml.	720	322	94	49	48	3	
Substrate	Galactose	Xylose	Mannitol	Adonitol	Erythritol	Rhamnose	Glycerol
μl. CO_2 per 2 ml.	-17	-25	-177	-207	-252	-252	-355

of experiments. There was an evolution of CO_2 from the substrates shown in the upper part of the table and an uptake in the case of substrates shown in the lower part. The actual comparative utilization of CO_2 is difficult to determine owing to variation in the relative state of oxidation of the substrate. The following three equations illustrate this point:



$$(\text{O.R.}=1).\quad 3CH_3COCOOH + H_2O = CH_3CH_2COOH + 2CH_3COOH + 2CO_2.$$

$$(\text{O.R.}=0).\quad 3CH_3CHOHCOOH = 2CH_3CH_2COOH + CH_3COOH + CO_2 + H_2O.$$

$$(\text{O.R.}=-1).\quad CH_2OHCHOHCH_2OH = CH_3CH_2COOH + H_2O.$$

If pyruvic acid which has a redox (O.R.) value of 1, is dissimilated to propionic acid, acetic acid and CO_2, one obtains 2 mol. of CO_2 for each 3 of pyruvic acid. In the case of lactic acid with an O.R. value of 0, 1 mol. of CO_2 is obtained from each 3 of lactic acid. Glycerol has a negative O.R. value and a balanced equation is obtained in this case in which no CO_2 is formed. Obviously, if CO_2 is produced in one reaction of the dissimilation and utilized in another, utilization will be apparent only if the uptake is greater than the production. In the case of pyruvic and lactic acids, the observed CO_2 formation was in approximate agreement with that calculated from the equations and probably little CO_2 was utilized. Glyceraldehyde, glucose, dihydroxyacetone and galactose are either 3- or 6-carbon compounds of the same oxidation-reduction level as lactic acid. A quantity of CO_2 equivalent to that from lactic acid is to be expected in these cases if there is no fixation of CO_2 and the dissimilation of the compounds is complete; the decrease in the rates of CO_2 evolution indicated that the substrates were completely fermented. Consequently the observed decrease in the production of CO_2 as compared with lactic acid (Table 1) may be an approximation of the amount of fixation of CO_2 occurring in the dissimilation of these substrates. Of course, conversions other than those expressed in the equation for lactic acid dissimilation (apart from CO_2 utilization) may take place, but qualitatively the above conclusions are undoubtedly true. In the case of the reduced compounds mannitol, adonitol, erythritol, rhamnose and glycerol, the formation of CO_2 is less than the utilization and the net result is the observed CO_2 uptake. These results prove that there is fixation in the dissimilation of a number of compounds (those in the lower part of Table 1) and indicate that CO_2 may have been utilized with the other substrates although an actual CO_2 uptake could not be demonstrated.

Comparative utilization of CO_2 under CO_2 and N_2. The utilization of CO_2 might be expected to increase with concentration of CO_2, and in fact this is shown to be true with a number of substrates (Table 2). CO_2 in the fermentation under N_2 was absorbed in alkali and thus was continuously removed from the medium, whereas in the comparative experiment the medium was saturated with CO_2. A survey of the results in Table 2 shows with each substrate that the formation of CO_2 was less or utilization was greater with an atmosphere of CO_2 than with N_2. The CO_2 values are expressed as m*M* per 100 m*M* of substrate and therefore are on a comparable basis. Differences between the CO_2 values under N_2 and under CO_2 probably represent roughly the change in CO_2 utilization under the two conditions. These experiments offer additional evidence that CO_2 is utilized in the dissimilation of a number of substrates with which a direct CO_2 uptake could not be demonstrated. It is possible that these changes in CO_2 values are caused by some change in the mechanism of dissimilation other than CO_2 utilization but this explanation is not supported by present evidence.

Inhibition of fixation of CO_2. In order to obtain information which might be more readily applicable to the study of tissues, a number of physiologically active inhibitors were tested for their effects on CO_2 utilization in the dissimilation of glycerol (Table 3). Fluoride, iodoacetate, malonate, azide, arsenite, cyanide and pyrophosphate were tested. Malonate and pyrophosphate were used especially because they inhibit succinic dehydrogenase and arsenite because of its effect on decarboxylation of β-keto-acids. A possible mechanism accounting for CO_2 utilization as well as succinic acid formation involves the addition of CO_2 to pyruvic acid to form oxaloacetic acid, followed by reduction to malic acid, dehydration to fumaric acid and finally reduction to succinic acid. Neither malonate nor pyrophosphate affected utilization of CO_2. The values obtained were 29·7 m*M* and 23·9 m*M* compared with 29·8 m*M* with no addition of inhibitor. It is possible that the process was stopped at fumaric acid. Arsenite might be expected to prevent the formation of oxaloacetic acid. However, this does not seem probable, for 0·04 *M* arsenite did not inhibit the breakdown of pyruvic acid by propionic acid bacteria. These points will be considered in a subsequent report.

It is of interest that cyanide and azide inhibit photosynthesis but are without effect on fixation of CO_2 by the propionic acid bacteria. This observation might lead one to suggest that the two processes do not involve similar reactions. However, the fixation of CO_2 in photosynthesis is closely associated with respiration. It is possible that the same process occurs in the fixation of CO_2 in the dark by propionic acid bacteria as in photosynthesis and the cyanide and azide inhibit photosynthesis by their action on the respiratory system.

Only NaF and iodoacetate of the group of inhibitors tested inhibited CO_2 utilization. Iodoacetate was not very suitable, however, because it likewise suppressed the entire dissimilation when used in effective concentrations, only 36·7 % of the glycerol being fermented. NaF is quite satisfactory. When no NaF was added, 29·8 m*M* of CO_2 were utilized per 100 m*M* of glycerol. A concentration of 0·000625 *M* NaF reduced the utilization of CO_2 to 8·8 m*M* and with 0·00125 *M* NaF utilization was prevented (1·5 m*M*) although the dissimilation was by no means completely inhibited. Table 4 shows the comparative effects of fluoride on the utilization of CO_2 with a number of substrates. In each dissimilation utilization of CO_2 was apparently inhibited although galactose and arabinose were not greatly affected probably because there was little utilization of CO_2. In similar experiments (Table 2) in which CO_2 utilization probably occurred, only 15·8 and 19·7 m*M* of CO_2 were formed from galactose and

Table 2. *Comparative formation of CO_2 from various substrates by cell suspensions of* P. pentosaceum *(49 W) under CO_2 and under N_2*

Bacteria with no substrate took up 32 μl. of CO_2 under CO_2 and produced 13 μl. under N_2

Substrate	Glucose		Galactose		Arabinose		Dihydroxy-acetone		Mannitol		Adonitol		Glycerol	
Atmosphere	N_2	CO_2	N_2	CO_2	N_2	CO_2	N_2	CO_2	N_2	CO_2	N_2	CO_2	N_2	CO_2
% substrate fermented	97·5	96·1	73·5	67·1	55·4	49·2	99·3	97·3	31·0	32·7	83·0	81·0	93·0	94·2
μl. CO_2 per 2 ml.	177	106	83·5	26·5	74·5	29	97·0	77·5	33	−22	0·0	−114	0·0	−216
mM CO_2 per 100 of fermented substrate	36·5	21·8	45·7	15·8	45·1	19·7	39·2	21·5	21·5	−13·6	0·0	− 23·8	0·0	− 23·5

Table 3. *Effect of inhibitors on utilization of CO_2*

Inhibitor	None	NaF		Iodoacetate		Malonate	NaN_3	$NaAsO_2$	KCN	Pyrophosphate
Molarity of inhibitor	—	0·00125	0·000625	0·000312	0·000078	0·03	0·002	0·01	0·1	0·02
% glycerol fermented	90·0	62·0	90·2	36·7	48·5	81·5	94·2	93·7	80·1	85·2
μl. CO_2 per 2 ml.	−261	− 9	−77	−35	−82	−235	−216	−280	−172	−198
mM CO_2 utilized per 100 of fermented glycerol	29·8	1·5	8·8	9·8	17·3	29·7	23·5	30·7	22·1	23·9

Table 4. *Effect of NaF on the utilization of CO_2 from various substrates*

Substrate	Glycerol		Adonitol		Mannitol		Glucose		Galactose		Arabinose	
Molarity of NaF	None	0·001	None	0·001	None	0·001	None	0·001	None	0·00125	None	0·001
% substrate fermented	99·3	90·8	94·3	97·0	94·3	48·6	99·3	90·1	98·6	92·7	79·1	69·1
μl. CO_2 per 2 ml.	−241	−20	−152	117	46	89	55	151	316	364	191	178
mM CO_2 per 100 of fermented substrate	− 22·3	− 2·3	27·3	20·5	9·9	37·2	22·2	67·5	64·3	78·8	40·4	43·1

arabinose as compared with 67·5 and 40·4 in the present experiment. In the case of glucose with no NaF present, 22·2 m*M* of CO_2 were formed. When CO_2 utilization was inhibited by NaF, the production of CO_2 increased to 67·5 m*M*. Assuming that the observed CO_2 is the net result of both formation and utilization of CO_2, this increase is to be expected since uptake of CO_2 was eliminated. Although the only infallible proof of CO_2 utilization lies in a direct demonstration of CO_2 uptake, the increased production of CO_2 in the presence of a known inhibitor is strong evidence of utilization, even in those dissimilations not affording direct proof.

In preparation for quantitative investigations involving determination of all products of dissimilation, a stronger buffer was used so that more substrate would be fermented. The results (Table 5) show the effect of increasing concentration of NaF on the utilization of CO_2 in the dissimilation of glucose (upper part of table) and glycerol (lower part of table) with 0·72 % $NaHCO_3$ and 1·6 %

Table 5. *Effect of increasing concentration of NaF on the utilization of CO_2 in glucose and glycerol dissimilations*

0·05 g. wet bacteria per ml.

NaF *M*	Substrate fermented %	CO_2 per ml. μl.	CO_2 per 100 m*M* of substrate m*M*
	Time = 20 hr.		
0·72 % $NaHCO_3$		0·8 % glucose	
0·000	100·0	287	30·0
0·001	99·6	349	36·6
0·005	97·3	352	37·8
0·0075	93·1	387	43·4
0·010	79·0	426	56·3
0·015	42·5	—	—
0·020	15·6	—	—
	Time = 30 hr.		
1·44 % $NaHCO_3$		1·6 % glucose	
0·000	99·8	556	28·0
0·001	99·5	586	29·6
0·0025	98·9	821	41·7
0·005	96·8	884	45·9
0·010	95·2	998	52·6
0·015	78·1	1034	66·5
0·020	65·9	812	61·9
	Time = 33·5 hr.		
0·72 % $NaHCO_3$		0·8 % glycerol	
0·000	95·7	-647	-34·5
0·005	75·7	-263	-17·7
0·0075	69·9	-219	-16·0
0·010	62·8	- 66	- 5·4
0·015	44·3	+ 24	2·8
	Time = 45·5 hr.		
1·44 % $NaHCO_3$		1·6 % glycerol	
0·000	78·7	-1594	-52·0
0·005	77·5	-1468	-48·6
0·0075	75·2	-1254	-42·8
0·010	69·8	- 941	-34·6
0·015	41·5	- 284	-17·5

substrate. The inhibition of fixation of CO_2 by increasing concentrations of NaF is clearly shown in the case of glycerol. The fixation decreased from 34·5 m*M* to a production of 2·5 in the one case and from 52·0 m*M* to 17·5 in the other case with increase in concentration of NaF. With glucose, as fixation of CO_2 was inhibited, the yield of CO_2 increased from 30·0 to 56·3 and from 28·0

to 61·9 mM. However, the effective molarity is much higher in $NaHCO_3$ buffer than in phosphate buffer. With 0·72 % $NaHCO_3$ *c.* 0·010 M NaF was necessary effectively to stop CO_2 utilization, whereas with 1·4 % $NaHCO_3$, 0·015 M NaF was required. This concentration of NaF is approximately ten times that required to inhibit utilization of CO_2 with phosphate buffer (Table 3). The *p*H values of the respective buffers after saturation with CO_2 were: (1) phosphate buffer 0·1 M (21 ml. 0·1 M K_2HPO_4 and 79 ml. 0·1 M KH_2PO_4), *p*H 5·8; (2) 0·72 % $NaHCO_3$, *p*H 6·6; (3) 1·4 % $NaHCO_3$, *p*H 6·8.

Lipmann [1929] has shown that inhibition of glycolysis by NaF is a function of *p*H. Apparently the same phenomenon occurs in inhibition of fixation of CO_2 by NaF.

The fact that both utilization of CO_2 and fermentation of phosphoglycerate are inhibited by NaF suggests a relationship of the two processes. In this case the effective concentration of NaF should be identical in the two processes. Table 6 shows the effect of increasing concentration of NaF on the dissimilation

Table 6. *Effect of increasing concentration of NaF on the dissimilation of phosphoglycerate*

Molarity of NaF	0·0000	0·0004	0·0007	0·0015	0·0050	0·0100
μl. CO_2 per 2 ml. from phosphoglycerate	341	312	306	184	86	91
μl. CO_2 per 2 ml. (endogenous)	59	66	66	59	76	90
Difference	282	246	240	125	10	1

Reaction mixture contained 0·225 % phosphoglyceric acid (adjusted to *p*H 6·9), 0·72 % $NaHCO_3$, 0·050 g. wet bacteria per ml. and varied concentration of NaF. Atmosphere CO_2. Reaction time 18 hr. Bacteria were harvested from 6 days' growth in medium containing glucose 0·5 %, $NaHCO_3$ 1·0 % and yeast extract (Difco) 0·4 %.

of phosphoglycerate on the basis of CO_2 evolution. A molarity of 0·005 almost completely stopped the dissimilation of phosphoglyceric acid. This concentration of NaF only partially stopped utilization of CO_2 and 0·01 M NaF was necessary for substantially complete inhibition (Table 5). These results leave some question as to whether the same reaction is being affected in the two processes. The nature of the experiments precludes an exact duplication of experimental conditions and the observed differences in the concentrations of NaF may be caused by some uncontrolled factor. A greater concentration of NaF is required to inhibit the dissimilation of phosphopyruvic acid than phosphoglyceric acid by muscle (0·01 M inhibits phosphoglyceric acid 100 % and phosphopyruvic acid 20 % [Lohman & Meyerhof, 1934]). Possibly CO_2 utilization involves the latter compound. The effect of iodoacetate on utilization of CO_2 may be due to the inhibition of pyruvic acid breakdown. The activity of NaF indicates that utilization of CO_2 has a close connexion with phosphorylation. Further evidence for this suggestion is that phosphate is necessary for optimal CO_2 utilization and succinic acid formation [cf. Wood & Werkman, 1940]. From the standpoint of thermodynamics this concept seems reasonable since the high energy content of the phosphorylated compounds may be the source of energy for CO_2 uptake.

SUMMARY

The fixation of CO_2 by cell suspensions of *Propionibacterium pentosaceum* has been investigated using a variety of substrates. The dissimilation of mannitol, adonitol, erythritol, glycerol and rhamnose under an atmosphere of CO_2 was accompanied by a definite uptake of CO_2, whereas glyceraldehyde, glucose,

dihydroxyacetone, arabinose, galactose and xylose gave a small evolution or some uptake. The evolution of CO_2 from lactic and pyruvic acids was much larger. Dissimilation of most of the substrates in an atmosphere of N_2 occurred with an evolution of CO_2 which was usually much larger than in an atmosphere of CO_2, indicating that utilization of CO_2 is proportional to the CO_2 concentration. These results suggest that the fixation of CO_2 may be a phenomenon of widespread occurrence.

Malonate, azide, arsenite, cyanide and pyrophosphate had no influence on CO_2 fixation. NaF and iodoacetate inhibit CO_2 utilization but iodoacetate is not satisfactory because it suppresses the entire dissimilation. NaF increased the evolution of CO_2 from a number of substrates. This fact is further evidence of its utilization in their dissimilation. The concentration of NaF necessary to inhibit dissimilation of phosphoglyceric acid was about one-half that required to inhibit utilization of CO_2. The reactions inhibited may not be identical in the two processes. It is probable, however, that a phosphate ester, possibly phosphopyruvic acid, is involved in the utilization of CO_2.

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