XXV. THE EFFECT OF CO_2 ON THE PRODUCTION OF SUCCINIC ACID BY *BACT. COLI COMMUNE*

By SIDNEY REUBEN ELSDEN

From the Biochemical Laboratory, University of Cambridge

(*Received 13 November 1937*)

Two theories have been proposed to explain the production of succinic acid during the fermentation of carbohydrates and allied substances by bacteria. The first, suggested by Grey [1924], supposed that in fermentations by *Bact. coli* succinic acid arose by the condensation of two molecules of acetic acid in the presence of a suitable hydrogen acceptor to give one molecule of succinic acid and two atoms of hydrogen. Recently Wood & Werkman [1936] have shown that the propionic acid bacteria form succinic acid from glycerol, and they suggest that it arose from acetic acid.

The second theory was put forward by Virtanen [1925, 1934], and by Virtanen & Karström [1931]. This postulated a cleavage of the hexose molecule into a 4C compound and a 2C compound. The 4C compound was supposed to be changed into succinic acid by an intramolecular rearrangement. Sheffer [1928] constructed carbon balance sheets for the fermentation of glucose by various species of the coli-aerogenes group. He deduced from his results that succinic acid could arise only in the manner suggested by Virtanen.

There have been, as yet, no decisive experiments to test these theories but, during the past year or so, a number of facts have come to light which may have some bearing on the problem of the anaerobic formation of 4C dicarboxylic acids by bacteria. Wood & Werkman [1936] observed that during the fermentation of glycerol by certain species of the propionic acid bacteria there was a fixation of CO_2. They were unable to decide in what form the fixed CO_2 appeared, but suggested that it acted as a hydrogen acceptor in some reaction. They pointed out however that where there was a large utilization of CO_2 a large amount of succinic acid was formed.

In this communication it will be shown that, under strictly anaerobic conditions, the rate of succinic acid formation by washed suspensions of *Bact. coli commune* from sodium pyruvate, glucose and galactose is dependent on the concentration of CO_2 in the medium.

Methods

(1) *Preparation of the suspension.* The strain of *Bact. coli commune* used in this work was provided by the Bacteriological Dept., Sheffield University, through Dr H. A. Krebs. The organism was grown on the surface of meat broth agar, in Roux bottles. After incubation for 14–16 hr. at 38°, the bacteria were washed three times at the centrifuge with distilled water and finally suspended in saline containing $M/20$ bicarbonate [Krebs & Henseleit, 1932], or in $M/20$ phosphate buffer. The dry weight of the organism per ml. was determined by means of a photoelectric turbidimeter [Clifton *et al.* 1935]. All experiments were carried out at 38°.

(2) *The estimation of succinic acid.* A manometric method for the determination of succinc acid involving the use of the succinic dehydrogenase of heart muscle was used. A similar method, based on the same principle, has recently been described by Weil-Malherbe [1937].

The enzyme was prepared afresh each week from pigs' heart by the method of Ogston & Green [1935]. The precipitate obtained from the phosphate buffer extract by adjusting the *p*H to 4·6 gave a homogeneous suspension in 20–25 ml. of phosphate buffer *p*H 7·2. Cresyl blue was the carrier used [see also Weil-Malherbe, 1937]. The wet preparation, like the dry preparation from ox heart used by Weil-Malherbe, oxidized only succinic acid, α-glycerophosphate and α-hydroxyglutaric acid.

The details are due mainly to Dr H. A. Krebs (private communication) and were as follows. The fluid to be examined for succinic acid was brought to *p*H 1–2 with 50% sulphuric acid and extracted for 4 hr. with freshly distilled, dry ether. At the end of the extraction period 2 ml. of *M*/10 phosphate buffer *p*H 7·2 were added to the ethereal extract and the ether distilled off, the succinic acid remaining behind in the buffer. This latter was evaporated down on a water bath to about 0·5 ml. and transferred to a 5 ml. measuring cylinder with *M*/20 phosphate buffer *p*H 7·2. The *p*H was adjusted to about 7·2 with *N*/10 NaOH. The final volume was usually between 2·5 and 3·5 ml. 2 ml. of this solution were added to the main compartment of the manometer cup with 0·1 ml. of a 0·5% solution of cresyl blue. Filter paper-KOH absorbers were used in the centre tube; 0·8 ml. of the enzyme preparation placed in the side bulb was tipped in after equilibration. An enzyme blank was set up with 2 ml. of *M*/20 phosphate buffer *p*H 7·2 instead of the extract. The oxidation was considered complete when the oxygen uptake fell to a value equal to the blank oxygen uptake over a period of 20 min.

Ethereal extraction, although increasing the time required for the estimation, was used for the following reasons: (*a*) A larger amount of succinic acid may be taken for the final estimation owing to the greater concentration, with a consequent gain in accuracy. (*b*) The possible presence of coenzyme would cause substances other than succinic acid (lactic acid, malic acid formed from the fumaric acid) to be oxidized. Coenzyme I is insoluble in ether. (*c*) The estimation of α-glycerophosphoric is prevented since this substance is also insoluble in ether.

Recovery experiments show an accuracy of about 95% (succinic added, 140 μl.; recovered 132 μl., 136 μl., duplicates).

(3) *The estimation of pyruvic acid.* Pyruvic acid was estimated by the carboxylase method of Westerkamp [1933] with the modifications introduced by Krebs & Johnson [1937]. The brewer's yeast was dried at 30° by means of a hot air drier.

(4) *Glucose and galactose* were estimated by the method of Hagedorn and Jensen. For the latter substance the tables published by Gale [1937] were used.

Metabolic quotients

The usual manometric conventions have been adopted for the expression of the rates of metabolic changes, the amounts of metabolites being expressed as μl. (1 m.mol. = 22,400 μl. at N.T.P.). The general formula for the metabolic quotients used is as follows.

$$Q_{\text{metabolite}} = \frac{\mu\text{l. of metabolite}}{\text{mg. dry weight of bacteria} \times \text{hr.}}.$$

Q_s refers to $Q_{\text{succinic acid}}$.

Experimental

(1) *Fermentation in high concentrations of CO_2.* The only substance tested which increased the rate of succinic acid formation from pyruvate was CO_2. Addition of lactate, acetate or formate gave negative results (Table I). In these experiments the total succinic acid formed, and not the rate of succinic acid formation, was measured.

Table I. *Effects of lactate, acetate, formate and CO_2 on the total succinic acid formed from pyruvate*

Warburg manometers, final volume of fluid in the cup 4 ml. The bacteria were suspended in saline containing *M*/20 bicarbonate. 2796 μl. of pyruvate were taken in each experiment and 0·5 ml. *M*/10 solution of the other substrates. Gas phase, unless otherwise stated, N_2/CO_2.

Exp. no.	Substrate	Succinic acid formed μl.
1	Pyruvate	261
	Pyruvate + lactate	274
	Lactate + acetate	0
	Pyruvate + acetate	169
2	Pyruvate	284
	Pyruvate + formate	253
	Pyruvate	263
	Pyruvate (pure CO_2 gas phase)	322

Each Warburg manometer had 3 ml. of the suspension of the organisms in bicarbonate saline. The substrates were tipped in from the side bulb at the end of 20 min. equilibration. The final volume in each cup was 4 ml. The gas phase was either nitrogen containing 5% CO_2 or pure CO_2. Unless otherwise stated N_2/CO_2 gas mixture implies nitrogen containing 5% CO_2. Anaerobiosis was secured by the use of freshly scraped sticks of yellow phosphorus placed in the centre tube of the manometer cup. A blank to which no substrate was added was set up. If the rate of succinic acid formation was being determined, the reaction was stopped at the end of 1 hr. by the addition of 0·5 ml. 50% H_2SO_4; if, however, the total succinic acid formed was being investigated the incubation was continued until the rate of gas evolution in the experimental manometer had fallen to that in the blank. When this occurred the reaction was stopped as described above. The contents of the cups with 0·5 ml. additional H_2SO_4 and 3 ml. of washings were transferred to the extractors for the extraction of the succinic acid.

Raising the concentration of CO_2 in the gas phase increases the total amount of succinic acid formed (Table I). The rate of succinic acid formation is also increased by raising the partial pressure of CO_2 in the gas phase (Table II).

Table II. *Effect of varying the CO_2 concentration on the rate of succinic acid formation from pyruvate*

Volume of fluid 4 ml. Bicarbonate concentration *M*/20. 3366 μl. of pyruvate were used in each experiment and the time was 60 min.

Exp. no.	N_2/CO_2 gas phase		CO_2 gas phase	
	Blank	Experimental	Blank	Experimental
1	$Q_s+1·3$	$Q_s+14·0$	$Q_s+0·98$	$Q_s+21·1$
2	$Q_s+1·6$	$Q_s+20·7$	Q_s 0·0	$Q_s+29·4$
3	Q_s 0·0	$Q_s+18·9$	Q_s 0·0	$Q_s+31·2$

Increasing the concentration of CO_2 in the gas phase has two effects on the fermentation fluid; (*a*) it increases the amount of dissolved CO_2 and (*b*) it lowers the *p*H. At 38° an *M*/20 solution of bicarbonate in equilibrium with nitrogen containing 5% CO_2 has a *p*H of 7·8; whereas the same solution with pure CO_2 has a *p*H of 6·5. Thus the observed increase in the rate of succinic acid formation may be due either to the increase in the amount of dissolved CO_2, or to the fall in *p*H. It is difficult to arrange experiments with the CO_2-bicarbonate buffer to test this point. For a 20-fold increase in the concentration of dissolved CO_2 there is only a 50–60% increase in the rate of succinic acid formation; suggesting that, if CO_2 is utilized by the organism in the synthesis of succinic acid the enzyme or enzymes concerned in its fixation must be almost saturated with their substrate when its concentration in the gas phase is only 5%. Glucose gave no clear-cut increase of succinic acid production when fermented in *M*/20 bicarbonate with a pure CO_2 gas phase.

(2) *Fermentation in low concentrations of CO_2.* If CO_2 is utilized by the organism to form succinic acid from pyruvate it would be expected that a reduction of the concentration of CO_2 would result in a corresponding reduction in the rate of succinic acid formation. In order to test this point the technique employed by Gladstone *et al.* [1935] when investigating the effect of CO_2 on bacterial growth was used. Nitrogen, carefully freed from CO_2 and oxygen, was bubbled through the fluid to remove the CO_2 as formed. Cylinder nitrogen was freed from CO_2 by passage through two wash bottles containing 15% NaOH, and from oxygen by passage over heated, palladized copper wire. The copper was reduced by heating in a current of hydrogen before use. In the first series of experiments the fermentation fluid was contained in a large boiling tube. The gas passed in through a Jena gas distribution tube. By means of a gas outlet the first fermentation tube was connected *via* a wash bottle containing 15% NaOH to a second fermentation tube containing water in place of substrate.

M/20 phosphate buffer *p*H 6·9 boiled just before use to remove dissolved gases was used in these experiments. The final volume in each tube was 40 ml., i.e. 30 ml. of the bacterial suspension and 10 ml. of the substrate or distilled water. The gas was passed through the suspension for 10 min. to equilibrate, the tubes were then momentarily disconnected and the substrate or water added. At the end of a given time the fermentation was stopped by the addition of 5 ml. of 50% H_2SO_4. The total volume of contents and three washings was usually 80–85 ml.; 15 ml. were taken for the estimation of succinic acid.

As a control, similar experiments in which the N_2 was replaced by the N_2/CO_2 gas mixture were carried out.

When N_2/CO_2 is replaced by N_2 the rate of succinic acid formation falls to approximately 40% of that in the N_2/CO_2 (Table III). In other words the removal of CO_2 from the fermentation fluid results in a decrease in the rate of succinic acid formation, as would be expected if CO_2 took part in the formation of succinic acid. Glucose gave similar results to pyruvate (Table III). Here the effect was more marked, the rate in N_2 falling to about 25% of that in N_2/CO_2.

After the above results had been obtained with pyruvate and glucose, Dr H. A. Krebs, in a personal communication, pointed out that galactose gave rise to large amounts of succinic acid when fermented in a bicarbonate-CO_2 buffer. This has been confirmed (Table IV). In addition experiments with galactose show particularly well the reduction in rate of succinic acid formation when CO_2 is rapidly removed from the medium (Table III). Here the rate falls to 0–13% of that in the control in N_2/CO_2.

Table III. *Effect of reducing the concentration of CO_2 in the fermentation fluid on the rate of succinic acid formation*

Time min.	Substrate	N_2 gas phase		N_2/CO_2 gas phase	
		Blank	Experimental	Blank	Experimental
45	Pyruvate, 10 ml. $M/5$	131 μl. $Q_s+2{\cdot}0$	462 μl. $Q_s+7{\cdot}2$	168 μl. $Q_s+2{\cdot}6$	1140 μl. $Q_s+17{\cdot}7$
60	Pyruvate, 10 ml. $M/5$	0 Q_s —	757 μl. $Q_s+6{\cdot}3$	152 μl. $Q_s+1{\cdot}3$	1880 μl. $Q_s+15{\cdot}7$
60	Glucose, 10 ml. $M/10$	0 Q_s —	257 μl. $Q_s+2{\cdot}4$	0 Q_s —	1230 μl. $Q_s+11{\cdot}8$
60	Glucose, 10 ml. $M/10$	85 μl. $Q_s+0{\cdot}9$	504 μl. $Q_s+5{\cdot}3$	102 μl. $Q_s+1{\cdot}1$	1867 μl. $Q_s+19{\cdot}4$
90	Galactose, 10 ml. $M/10$	0 Q_s —	0 Q_s —	0 Q_s —	1180 μl. $Q_s+8{\cdot}2$
60	Galactose, 10 ml. $M/10$	0 Q_s —	176 μl. $Q_s+1{\cdot}9$	158 μl. $Q_s+1{\cdot}7$	1250 μl. $Q_s+13{\cdot}7$

Table IV. *Total succinic acid formed from galactose*

$M/20$ bicarbonate in saline; 4 ml. total vol. in each cup; 1370 μl. of galactose in each experiment.

Blank μl.	Experimental μl.	Experimental − blank μl.	Mol. succinic acid per 100 mol. galactose
164	1115	951	73
127	1070	943	72
120	1228	1108	84·7
70	1193	1123	86

Under these conditions the shift in pH when changing from N_2 to N_2/CO_2 is very small. The pH of the CO_2-free phosphate ($M/20$) as determined by the quinhydrone method was 6·9; CO_2-free nitrogen was bubbled through during the determination. When N_2 was replaced by N_2/CO_2 the pH fell to 6·75. This change is insignificant, more especially as there is a continuous production of free acid during the fermentation.

(3) *The effect of reducing the CO_2 concentration on the rate of substrate utilization.* It might be argued that CO_2 acts by simply increasing the rate of substrate utilization and consequently does not play a direct part in the formation of succinic acid. The observed decrease in the Q_s would then be due to the decreased rate of fermentation in the absence of CO_2.

The rate of substrate utilization under the conditions of the previous set of experiments was therefore determined. This necessitated a modification of the technique which, as it stood, was unsatisfactory when substrate disappearance had to be estimated. The chief objection to the technique was that the final volume of the fluid was so great that the figures for the substrate remaining had to be multiplied by a factor of at least 80, as only 1 ml. samples could be taken; whereas the figures for succinic acid had to be multiplied by a factor of about 5.

An apparatus which would allow working on a much smaller scale was designed. This consisted of a small boiling tube fitted with a side bulb. The gas was passed in through a Jena sintered glass filter stick and escaped through a tube attached to the side bulb. 3 ml. of the suspension containing not more than 20 mg. dry weight of bacteria were used; if thicker suspensions were

employed foaming occurred, and this resulted in a loss of some of the fluid through the outlet tube. 1 ml. of substrate was placed in the side bulb.

The gas was passed through the fluid for 10 min. to equilibrate, the tube disconnected and the substrate tipped into the main compartment. The fermentation was stopped by immersing the tube in a boiling water bath for 5 min. The contents were transferred to a 15 ml. graduated flask and the volume made up to the mark with the washings of the tube. 10 ml. were taken for the succinic acid estimation and the remainder kept for the determination of the amount of substrate unused. In these experiments no controls without added substrate were set up. The results obtained are summarized in Table V.

Table V. *Effect of reducing the CO_2 concentration on the rate of substrate utilization and succinic acid formation*

Time min.	Substrate	N_2 gas phase		N_2/CO_2 gas phase	
		$Q_{substrate}$	Q_s	$Q_{substrate}$	Q_s
60	Pyruvate, 7650 μl.	−266	+12·1	−282	+29·1
60	Pyruvate, 7650 μl.	−288	+ 9·8	−291	+25·6
60	Glucose, 2055 μl.	− 56·1	+ 1·9	− 58·2	+11·4
60	Glucose, 2055 μl.	− 61·2	+ 1·4	− 63·4	+ 8·9
120	Galactose, 992 μl.	− 5·7	0	− 8·1	+ 7·3
120	Galactose, 992 μl.	− 4·8	+ 0·6	− 6·6	+ 7·2

In the cases of glucose and pyruvate there is no significant change in the rate of substrate utilization when compared with the change in rate of succinic acid formation. Galactose on the other hand shows a decrease in the rate of disappearance when CO_2 is removed. This is insignificant when compared with the decrease in rate of succinic acid formation under the same conditions. The discrepancy in the galactose figures may be due to technical difficulties. Galactose was fermented extremely slowly and the amount used was small even though the runs lasted 2 hr. As the amount of galactose disappearing is obtained by difference, small errors are magnified.

The magnitude of the effect appears to vary inversely as the rate of fermentation of the substrate in question. Thus pyruvate is fermented more rapidly than glucose which in its turn is fermented more rapidly than galactose. On the other hand galactose responds more effectively to the removal of CO_2 than does glucose; and glucose shows the effect of removal of CO_2 more than does pyruvate. A possible explanation of this is that when a substrate is fermented rapidly there is a higher concentration of CO_2 in the cell than when a substrate is fermented slowly. And as the rate of removal of CO_2 is the same in both cases there will be a higher concentration of CO_2 in the cells in the presence of the more rapidly fermented substrates, and the effect of its removal will not be so marked.

Discussion and summary

The experiments quoted above, show that (*a*) when a washed suspension of *Bact. coli* ferments pyruvate, the rate of succinic acid formation is increased 50–60% by increasing the concentration of CO_2 in the medium; the total succinic acid formed under the same conditions is also increased; (*b*) that rapid removal of CO_2 from such a suspension fermenting pyruvate, glucose or galactose causes a decrease in the rate of succinic acid formation amounting to 40% of that in the presence of N_2/CO_2 in the case of pyruvate, 25% in the case of glucose and 13% or less with galactose. There is no significant decrease in the rate of substrate utilization when CO_2 is removed from the fermenting fluid.

These facts suggest that possibly CO_2 is involved in the synthesis of succinic acid. No direct evidence for the fixation of CO_2 by this organism has been obtained under the conditions used in these experiments and it would be very unwise to assume such a fixation on the basis of the data presented here. At the moment facts are too few even to permit theorizing on the role of CO_2 in succinic acid formation.

It should be remembered however that Woods [1936] has shown that under certain well-defined conditions *Bact. coli* is able to fix CO_2. These conditions were not fulfilled in this work. A further point of interest arises from the work of Gladstone *et al.* [1935], who showed that *Bact. coli* is unable to grow when CO_2 is rigidly excluded from the medium. The connexion between CO_2 as a growth factor for *Bact. coli* and CO_2 as a possible reactant in the anaerobic breakdown of certain hexoses and allied compounds is at the moment obscure.

The author wishes to express his gratitude to Dr M. Stephenson and to Dr D. D. Woods for their continual encouragement and advice; to Dr H. A. Krebs for much help with the manometric side of the work; and to Sir F. G. Hopkins for his interest in this research. The brewer's yeast used was kindly supplied by Messrs Greene, King and Son, Ltd.

This work was carried out during the tenure of a maintenance grant from the Department of Scientific and Industrial Research.

REFERENCES

Clifton, Mueller & Rogers (1935). *J. Immunol.* **29**, 377.
Gale (1937). *Biochem. J.* **31**, 234.
Gladstone, Fildes & Richardson (1935). *Brit. J. exp. Path.* **16**, 335.
Grey (1924). *Proc. roy. Soc.* B, **96**, 156.
Krebs & Henseleit (1932). *Hoppe-Seyl. Z.* **210**, 33.
—— & Johnson (1937). *Biochem. J.* **31**, 645.
Ogston & Green (1935). *Biochem. J.* **29**, 1983.
Sheffer (1928). Dissertation, Delft.
Virtanen (1925). *Soc. Sci. fenn., Comment. Physic. Math.* **2**, 20.
—— (1934). *J. Bact.* **28**, 447.
—— & Karström (1931). *Acta chem. fenn.*, Series B, **7**, 17.
Weil-Malherbe (1937). *Biochem. J.* **31**, 299.
Westerkamp (1933). *Biochem. Z.* **263**, 239.
Wood & Werkman (1936). *Biochem. J.* **30**, 48.
Woods (1936). *Biochem. J.* **30**, 515.