The Effect of Carbon Dioxide Pressure on a Bacterial Decarboxylase System

BY W. H. OLIVER*

The Dairy Research Institute (N.Z.), Palmerston North, New Zealand

SUMMARY: The effect of CO₂ on a decarboxylase system of *Streptococcus diacetilactis* has been investigated. It was found that the amount of CO₂ produced by a culture of the living organism depended upon the proportion of CO₂ present in the atmosphere. Under minimum p_{CO_2} the production of CO₂ by the organism was stimulated while accumulation of CO₂ repressed the formation of the gas. It also appeared that the presence of excess of other products of reaction repressed the production of CO₂. An explanation of these phenomena in terms of the law of mass action is advanced.

The effect of CO₂ upon the production of decarboxylase by the organism was also investigated. There was some indication that the composition of the gaseous environment influenced the production of decarboxylase.

During an investigation of the heat stability of an enzyme system of *Streptococcus diacetilactis* (Oliver, 1952) it was noticed that the amount of carbon dioxide produced by a culture of the organism varied according to whether the CO₂ was continuously removed or was allowed to accumulate under pressure in a closed culture apparatus. Where an enzymic reaction is readily reversible it is to be expected that the rate of reaction and the final end point will be influenced by accumulation of end products which, according to the law of mass action, will tend to drive the process in the reverse direction. Where one of the end products is a gas (as in the case under consideration) it may be allowed to escape into the atmosphere, as in a cotton-wool plugged flask; it may be removed from a closed system by absorption in alkali; or it may be confined in a sealed culture vessel with the production of pressures higher than atmospheric. The full implications of these variations in conditions of culture do not seem to have been appreciated by many workers who have investigated the production of CO₂ by plant and animal tissue and by micro-organisms, since manometric, gasometric, gas-entrainment and gas-absorption techniques have been used without any allowance for their possibly different effects on the course of the reactions studied.

In the present paper evidence is presented tending to show that with *Strep. diacetilactis* the equilibrium point of the enzymic reaction by which CO₂ is produced is influenced according to the law of mass action by CO₂ pressure, and further that the amount of enzyme formed by the organism may vary according to the gaseous environment of the growing culture.

MATERIALS AND METHODS

A culture of *Strep. diacetilactis*, strain DL, as described in a previous paper (Oliver, 1952) was used as the test organism, the culture media also being as described in that paper. The organism grew readily in sterilized skim milk

* Present address: New Zealand College of Pharmacy, Wellington, New Zealand.

at 22°, clotting the milk within 24 hr. with the formation of *c.* 0.8 % titratable acid (calculated as lactic acid). It should be emphasized that in all the experiments described, active multiplication of the *Strep. diacetylactis* ceased in 24–36 hr. and that the CO₂ production observed for the greater part of the experimental period was a result of the metabolic action of organisms maintained in a non-proliferating condition by the high acid concentration in the culture medium.

Carbon dioxide production was estimated as described in a previous paper (Oliver, 1952). Where it was desired to observe the effect of accumulation of CO₂ over the culture a short length of pressure tubing was fitted between the culture and baryta flasks so that the culture could be sealed off from the absorbent by a screw clip. When the clip was released the gases were bubbled through baryta solution and the CO₂ estimated in the usual manner. In this apparatus the volume of air available to the sealed-off culture was only half that available in the original apparatus where culture flask and absorption flask were open to one another from the start of the experiment. It was found, however, that p_{CO_2} is not critical for this particular enzyme system, for on excluding air by placing a layer of paraffin oil over the culture the total volume of CO₂ produced did not decrease, although the end point was reached a little later than under fully aerobic conditions.

RESULTS

Effect of CO₂ pressure on CO₂ production at 22°

Growth of *Strep. diacetylactis* at 22° in milk and in citrate broth in an apparatus in which CO₂ was continuously absorbed by 0.1 N-baryta gave volumes of CO₂ as recorded in Table 1. These figures represent the average result obtained in many experiments, there being only small variations from one culture to another. It will be noted that the total amount of CO₂ increased up to 10 days

Table 1. *Carbon dioxide produced by 100 ml. culture of Strep. diacetylactis at 22° in an atmosphere in contact with baryta initially 0.1 N (average figures)*

Medium	Days							
	1	2	3	4	5	6	7	10
	CO ₂ production (ml. N.T.P.)							
Milk	11	21	29	34	38	40	43	46
Broth + 0.01 M-citrate	13	30	41	48	52	54	55	56

(after which no appreciable increase occurred) and that gas production was higher in a citrate broth medium than in milk. The p_{CO_2} under these control experimental conditions (over 0.1 N alkali) is not accurately known, but it is very low since Dee (1945) derived a figure of 10^{-11} atmospheres for p_{CO_2} over a solution containing *c.* 0.25 N-NaOH + 0.25 N-Na₂CO₃.

When cultures of *Strep. diacetylactis* were grown in milk or in broth in a type of apparatus in which the culture flask was sealed off from the absorption

flask the volume of CO_2 produced was greatly reduced. Table 2 gives the results of a typical experiment in which culture flasks of two different sizes (containing the same volumes of culture) were used. It is clear that the sealing of the culture flasks decreased the CO_2 production as compared with the control, and that the smaller the gas space above the culture the greater the decrease in CO_2 production. As mentioned above, the volume of O_2

Table 2. *Carbon dioxide produced in 4 days at 22° by Strep. diacetylactis in 100 ml. of milk or citrate broth in sealed vessels of different sizes*

Medium	Gas space in sealed vessel		Unsealed control
	c. 40 ml.	c. 400 ml.	
	CO ₂ produced (ml. N.T.P.)		
Milk	2.0	7.9	35
Broth + citrate 0.01 M	5.7	19.8	47.5

available to the culture is not significant so far as the action of the CO_2 -producing enzyme is concerned. Furthermore, the density of the bacterial population and the total titratable acidity were found to be the same for all the cultures in a given medium irrespective of p_{O_2} or p_{CO_2} . This shows that the phenomenon is not due to a change in the rate of multiplication or in the main metabolic activity of the organism. The most reasonable hypothesis is that the equilibrium point of the enzymic reaction concerned in the formation of CO_2 is altered by CO_2 pressure according to the law of mass action.

Alteration of the equilibrium position by other end products

In a reaction of the type $\text{A} \rightleftharpoons \text{B} + \text{CO}_2$ the removal or addition of either B or CO_2 should affect the equilibrium in a similar way. In practice, it is easier to alter the concentration of the product in the gas phase (i.e. CO_2) than a product B in the liquid phase. Moreover, the exact course of the reaction by which diacetyl and CO_2 are produced by *Strep. diacetylactis* is not known, and hence the identity of B is unknown. However, it is reasonable to assume that the cell-free filtrate from the cultures will contain product B. On this assumption the effect on CO_2 production of adding Seitz-filtered whey, from a 3-day culture of *Strep. diacetylactis* grown over alkali, to a pasteurized culture of the same age and grown under the same conditions was examined. It had previously been shown (Oliver, 1952) that the decarboxylase system of the organism was stable beyond the temperature of pasteurization and would continue to function unabated after the organism had been killed. Thus the use of a culture killed by heating at 80° for 5 min. excluded the possibility that any observed decrease in CO_2 formation might be due to inhibition of the normal metabolic processes of the resting organism by the added products of its metabolism. Table 3 gives the results of a typical experiment in which a pasteurized milk culture of *Strep. diacetylactis* was incubated at 22° in a CO_2 -absorbing apparatus with and without the addition of Seitz-filtered

whely. It is clear that the presence of sterile whey in the pasteurized culture resulted in a decrease in CO₂ production to an extent depending on the proportion of whey added. The results are consistent with the hypothesis that metabolic products other than CO₂ are able to alter the equilibrium point of the reaction in which CO₂ is one of the end products.

Table 3. *Carbon dioxide produced by pasteurized 3-day milk cultures of Strep. diacetylactis with and without additions of Seitz filtered whey from a similar culture*

	CO ₂ produced in 4 days at 22° (ml. N.T.P.)
3-day culture heated at 80° for 5 min.	18.0
3-day culture + 5 ml. whey	11.1
3-day culture + 10 ml. whey	5.4
10 ml. whey in sterilized milk	2.0

Effect of CO₂ on the amount of decarboxylase produced by bacteria

Exposure of a growing culture of *Strep. diacetylactis* to carbon dioxide was found to influence the CO₂-producing capacity of the culture during a subsequent incubation period under conditions in which any CO₂ formed was immediately absorbed in baryta. Cultures in milk and in citrate broth were grown at 22° in three different sets of conditions; in flasks sealed with a screw clip, in flasks plugged with cotton wool, and in flasks attached to an absorption flask containing baryta. These cultures were thus under high, medium and minimum CO₂ pressure respectively during the preliminary growth period. After varying times under these conditions all the cultures were connected to absorption flasks containing alkali, and the incubation at 22° was continued until 7 days had elapsed from the start of the experiment. Table 4 gives a typical series of results. It will be observed that as compared

Table 4. *Carbon dioxide produced by Strep. diacetylactis at 22° after preliminary growth for various periods under various CO₂ tensions*

		Preliminary growth period (hr.)				
		18	24	48	72	96
		CO ₂ produced in subsequent 4 days at 22° (ml. N.T.P.)				
Conditions of preliminary growth	Medium					
Sealed flasks	Milk	28.1	10.9	3.0	1.7	0.6
	Broth	38.4	14.3	3.2	1.8	Nil
Cotton wool plugged flasks	Milk	31.3	22.5	15.0	9.7	5.0
	Broth	44.2	32.3	14.0	5.0	2.4
Control connected to CO ₂ absorption flasks from beginning	Milk	34.3	32.6	21.8	13.0	8.0
	Broth	46.7	42.7	24.2	14.0	7.5

with the control values (those obtained from the cultures grown throughout with continuous absorption of CO₂) the values for cultures grown under medium and high p_{CO_2} during a preliminary period were always significantly

lower, indicating that pressure of CO_2 on a culture tends to decrease its capacity for CO_2 production even when the pressure is subsequently removed.

These results suggest that the effect of CO_2 on *Strep. diacetylactis* is to inhibit synthesis of the decarboxylase. Under optimal conditions a smaller amount of catalyst should finally give the same total CO_2 production as a larger amount, although in a longer time, but in bacterial cultures in which various metabolic products have accumulated the enzymes suffer gradual destruction during the course of their action and hence the volume of CO_2 produced should correspond in some degree with the amount of enzyme formed by the organism.

The concept of evocation of specific enzyme activities in micro-organisms in response to variations in environment is accepted by many workers. Hauka & Koessler (1924) suggested that micro-organisms produce amino-acid decarboxylases in response to an over-acid environment 'as a protective measure when accumulation of hydrogen ions within the organism's protoplasm is incompatible with its normal life process'. Gale (1946) suggested that, since CO_2 is sometimes an essential metabolite, decarboxylases may be formed and produce CO_2 in growth conditions where retention of CO_2 in a medium is limited by solubility considerations. A strongly acid culture, such as is produced by *Strep. diacetylactis* in 24–36 hr., from which CO_2 is being continuously withdrawn fulfils these conditions for increased production of decarboxylase. The increased amount of enzyme formed during the earlier period would then be expected to result in a greater CO_2 production during the resting period. Conversely, a culture in which p_{CO_2} is allowed to increase during the early growth period may be expected to decrease its production of decarboxylase, this being reflected subsequently in a diminished CO_2 output.

It would obviously be desirable to investigate the problem further, using cell-free extracts from bacterial material obtained by centrifuging cultures grown under various CO_2 tensions and to compare decarboxylase activity under conditions of known substrate concentration. This has not been possible so far, in the absence of a satisfactory technique for preparing an active extract. Several methods of grinding centrifuged bacteria were tried, but it was found that the extracts obtained lost their decarboxylase activity within a few minutes. Evidently the decarboxylase was very unstable apart from the whole bacteria cell, or in a medium differing greatly from that in which it was produced.

DISCUSSION

Although studies have been made of the effect of accumulation of CO_2 in a closed system on the respiration of plant tissues and on the metabolic activities of micro-organisms little attention has been paid to the techniques used for estimation of CO_2 in such experiments. Willaman & Beaumont (1928), working with plant tissues, found that the accumulation of CO_2 resulted in a logarithmic decrease in CO_2 output. Hassouna & Allen (1939) investigated the gas production of lactic acid bacteria using a gasometric and an absorption technique. In some cases they observed no evolution of gas with the gaso-

metric method while the absorption technique recorded a significant CO₂ production. They attributed this anomaly to the solubility of CO₂ in the medium but it may well have been due to suppression of CO₂ formation in the gasometric apparatus. Osterwalder & Jenny (1939) found that the fermentation of wine was stopped by the accumulation of CO₂ in a closed vessel but, because they found that an increase in total pressure had a similar though smaller effect, they did not consider p_{CO_2} as having a specific effect on the fermentation. Increased atmospheric pressure results in a higher p_{CO_2} at the site of reaction, so an explanation in terms of the law of mass action is valid. Kidd & West (1945) reported that 10 % of CO₂ in the atmosphere suppressed CO₂ production in apples by 45 %. Zhuravskii (1939) observed suppression of citric acid formation and of mycelial growth by an atmosphere containing 20 % of CO₂. A pathway commonly postulated for citric acid formation (Sumner & Somers, 1947) involves condensation of oxaloacetate and pyruvate to form an intermediate which is oxidized and decarboxylated to citrate, so it might be expected that a high p_{CO_2} would suppress the last (decarboxylative) stage of the reaction.

Foster & Davis (1949) found 'that high CO₂ concentrations specifically inhibit fumarate formation under the above conditions (CO₂ fixation via the Wood-Werkman reaction) and that oxaloacetate decarboxylase activity of the mold is also inhibited by high CO₂ tensions, suggesting that fumarate formation is blocked at the pyruvate + CO₂ stage'. Suppression by CO₂ of decarboxylation by isolated oxaloacetate decarboxylase is to be expected on the basis of the mass law so that the term 'specific inhibition' of the decarboxylation is unnecessary. The scheme of reactions proposed by Foster & Davis for anaerobic consumption of glucose by *Rhizopus nigricans* involves a preliminary conversion to pyruvate, which may then be reduced in one of three ways: without decarboxylation, yielding lactate; after decarboxylation, yielding ethanol; and after carboxylation, yielding fumarate via malate. A high p_{CO_2} was also found to inhibit the production of ethanol, but not that of lactate, which is normally produced in small amount only. The substantial inhibition of ethanol formation and of glucose consumption in the presence of CO₂ may indicate that the whole series of reactions is being forced back via the route $\text{CH}_3\text{CHO} + \text{CO}_2 \rightleftharpoons \text{pyruvate} \rightleftharpoons \text{hexose}$. A knowledge of the energy requirements of the various reactions should be helpful in understanding why the pyruvate which is formed is converted to ethanol rather than to oxaloacetate. The suppression of alcoholic fermentation by CO₂ is often accompanied by a decrease in the rate of growth of the organism, this being in contrast with the effect on *Strep. diacetylactis* where growth is not retarded. In the former case ethanol and CO₂ are the end products of the main metabolic pathway and energy source, while in the latter the production of diacetyl and CO₂ results from a relatively unimportant side reaction and the main energy source (the lactic acid fermentation) does not involve CO₂ production.

It thus appears that, depending upon the reactions involved, CO₂ pressure in a closed system could influence the production of CO₂ and the growth of a micro-organism in the following ways: (i) suppression of CO₂ production

and of growth of the organism through a mass action effect where production of CO_2 is involved in the main reactions brought about by the organism in the medium; (ii) suppression of CO_2 production without suppression of growth through a mass action effect on a reaction which is not essential to the growth of the organism; (iii) suppression of growth of the organism due to a non-specific effect of CO_2 acting as an acid with recovery to normal on removal of pressure. Whichever applies in a given case it is obviously important that the method of estimating CO_2 shall be specified and that there shall be an appreciation of the possible effect of CO_2 pressure on the results obtained. In a large majority of cases it appears that the simplest hypothesis to explain the effect of CO_2 in a closed system is the law of mass action. Since synthesis of adaptive enzymes can be stimulated by the presence in the medium of appropriate substrates, it might be expected that enzyme synthesis would be suppressed by presence of excess of the products of reactions. This would apply equally to gases and to substances in solution. No observations on this point seem to have been made hitherto, but the evidence presented here, although not unequivocal, does lend support to this hypothesis.

The author gratefully acknowledges Dr H. R. Whitehead's helpful criticism and advice throughout the investigation.

REFERENCES

- DEE, T. P. (1945). The partial pressure of carbon dioxide over aqueous solutions containing caustic soda and sodium carbonate. *J. Soc. chem. Ind., Lond.* **64**, 1.
- FOSTER, J. W. & DAVIS, J. B. (1949). Carbon dioxide inhibition of anaerobic fumarate formation in the mold *Rhizopus nigricans*. *Arch. Biochem.* **21**, 135.
- GALE, E. F. (1946). The bacterial amino-acid decarboxylases. *Advanc. Enzymol.* **6**, 1.
- HASSOUNA, M. M. & ALLEN, L. A. (1939). Gas production by *B. coli* and by lactic acid streptococci under different conditions. *J. Dairy Res.* **10**, 7.
- HAUKA, M. T. & KOESSLER, K. K. (1924). Studies in proteinogenous amines. *J. biol. Chem.* **59**, 855.
- KIDD, F. & WEST, C. (1945). Effect of carbon dioxide on carbon dioxide production in absence of oxygen. *New Phytol.* **44**, 231.
- OLIVER, W. H. (1952). The stability of some bacterial enzymes toward heat and chemical bactericides. *J. gen. Microbiol.* **7**, 329.
- OSTERWALDER, A. & JENNY, J. (1939). Behaviour of yeast toward carbon dioxide and carbon dioxide pressure. *Landw. Jb. Schweiz*, **53**, 371.
- SUMNER, J. B. & SOMERS, G. F. (1947). *Chemistry and Methods of Enzymes*, 2nd ed. New York: Academic Press Inc.
- WILLAMAN, J. J. & BEAUMONT, J. H. (1928). The effect of accumulated carbon dioxide on plant respiration. *Plant Physiol.* **3**, 45.
- ZHURAVSKII, G. I. (1939). The gas exchange in *A. niger* during formation of citric acid. *Microbiology (U.S.S.R.)*, **8**, 414. Quoted in *Chem. Abstr.* 1941, **35**, 484.

(Received 7 April 1952)