

- Foster, W. E. (1940). Ph.D. Thesis: Durham University.
 Harrison, A. (1953). Ph.D. Thesis: Durham University.
 Hulme, A. C. (1956). *Nature, Lond.*, **178**, 218.
 Leonard, O. A. (1945). *J. Amer. Soc. Agron.* **37**, 55.
 Magness, J. R. (1920). *Bot. Gaz.* **70**, 308.
 Ranson, S. L. (1953). *Nature, Lond.*, **172**, 252.
 Ranson, S. L. (1955). In *Modern Methods of Plant Analysis*, vol. 2, p. 539. Ed. by Paech, K. & Tracey, M. V. Berlin: Springer Verlag.
 Ranson, S. L., Walker, D. A. & Clarke, I. D. (1957). *Biochem. J.* **66**, 57 F.
 Russell, E. J. & Appleyard, A. (1915). *J. agric. Sci.* **7**, 1.
 Small, J. (1954). *Modern Aspects of pH*, chapt. 6. London: Baillière, Tindall and Cox.
 Smith, W. H. (1957). *Nature, Lond.*, **179**, 876.
 Thomas, M. (1925). *Biochem. J.* **19**, 927.
 Thornton, N. C. (1933). *Contr. Boyce Thompson Inst.* **5**, 403.
 Umbreit, W. W., Burris, R. H. & Stauffer, J. F. (1949). *Manometric Techniques and Tissue Metabolism*, chapt. 3. Minneapolis: Burgess Publishing Co.
 Ulrich, R. & Landry, J. (1956). *C.R. Acad. Sci., Paris*, **242**, 2757.
 Walker, D. A. & Beevers, H. (1956). *Biochem. J.* **62**, 120.
 Walker, D. A. & Brown, J. M. A. (1957). *Biochem. J.* **67**, 79.

Biochem. J. (1960) **76**, 221

Effects of Carbon Dioxide on the Oxidation of Succinate and Reduced Diphosphopyridine Nucleotide by *Ricinus* Mitochondria

BY D. S. BENDALL

Department of Biochemistry, University of Cambridge

S. L. RANSON

Department of Botany, King's College, Newcastle upon Tyne

AND D. A. WALKER

Department of Botany, Queen Mary College, London, E. 1

(Received 29 December 1959)

The oxidation of succinate to fumarate by particulate preparations from *Ricinus* endosperm is retarded in atmospheres containing more than about 10% of carbon dioxide (Ranson, Walker & Clarke, 1957, 1960) and the retardation has been attributed to an inhibition of the succinic-oxidase system of the mitochondria. The present paper is an enlargement of a preliminary account (Bendall, Ranson & Walker, 1958) and describes the results of an investigation to determine which components of the respiratory chain are inhibited. The effects of carbon dioxide-bicarbonate mixtures on the succinic-cytochrome *c* reductase system and on cytochrome *c* oxidase were determined, and evidence is presented that the actual site of inhibition is succinic dehydrogenase.

Since previous work had also suggested that the enzymic reoxidation of reduced diphosphopyridine nucleotide may be affected by high concentrations of carbon dioxide, the effects of carbon dioxide-bicarbonate mixtures on the reduced diphosphopyridine nucleotide-cytochrome *c* reductase system were also determined.

In an attempt to assess the effects of the sodium bicarbonate in the mixtures with carbon dioxide the effects of some other salts have also been studied.

MATERIALS AND METHODS

Cytochrome *c* was purchased from the Sigma Chemical Co., St Louis, Mo., U.S.A. and Boehringer und Söhne, Mannheim, Germany. It was dissolved in 1% (w/v) 2-amino-2-hydroxymethylpropane-1:3-diol (tris)-HCl buffer, pH 7.4, and estimated spectrophotometrically at 550 m μ . Molar extinction coefficients of 27.7×10^3 l. moles⁻¹ cm.⁻¹ for the reduced form and 9.2×10^3 l. moles⁻¹ cm.⁻¹ for the oxidized form were used (Margoliash, 1954). Reduced cytochrome *c* was prepared by adding a slight excess of sodium dithionite and bubbling O₂ through the solution for 5 min. to remove the excess of dithionite. Reduced diphosphopyridine nucleotide (DPNH) was purchased from the Sigma Chemical Co.

Preparations of mitochondrial fractions. Suspensions of mitochondria from the endosperm of castor beans (*Ricinus communis* var. Cimarron) were prepared as described previously (Ranson *et al.* 1960). 30 min. before spectrophotometer experiments the suspensions were diluted tenfold with ice-cold demineralized water, since it has been found that *Ricinus* mitochondria will not react easily with added cytochrome *c* when suspended in 0.5 M-sucrose but react readily after incubation in 0.05 M-sucrose. This is attributed to the osmotic rupture of membranes which would otherwise limit the free access of cytochrome *c* to enzymic centres.

Enzyme assays. Succinic-cytochrome *c* reductase, DPNH-cytochrome *c* reductase and cytochrome *c* oxidase activities were measured spectrophotometrically by

methods already described (Bendall *et al.* 1958) with a Unicam SP. 500 spectrophotometer in a temperature-controlled room at 25°.

Spectroscopic observations. These were made with a Zeiss microspectroscope ocular. The scale was set with the α -band of reduced cytochrome *c* at 550 $m\mu$.

Regulation of carbon dioxide concentration. Conditions in the aqueous reaction mixtures corresponding to various atmospheric CO_2 concentrations were simulated by the addition of CO_2 -bicarbonate mixtures as described (Ranson *et al.* 1960). The procedure was modified slightly for the spectrophotometer experiments in that the CO_2 concentration was regulated simply by the addition of the appropriate quantity of 0.316M- $NaHCO_3$ which had previously been brought to equilibrium at pH 7.4 and 25° with a gas mixture containing 90% of CO_2 and 10% of O_2 . The bicarbonate was added to the other components in closed spectrophotometer cells immediately before the reactions were started. Subsequent determinations of initial rates were completed within 2 min. Previous experience (Walker & Brown, 1956) had indicated that the loss of CO_2 from solution in these circumstances is extremely small.

RESULTS

Preliminary observations

The effects of a carbon dioxide-bicarbonate mixture on the succinic-oxidase system were examined spectroscopically as follows. To a 1 cm. cell containing 1.6 ml. of a suspension of washed mitochondria was added 0.4 ml. of 0.34M-sodium bicarbonate saturated with carbon dioxide to give a final concentration corresponding to approximately 20% of carbon dioxide. A control cell was set up in which 0.34M-sodium chloride replaced the bicarbonate solution. The cells were shaken with air and to each was added 0.5 ml. of 1.2M-potassium succinate. In the control mixture the α -bands of reduced cytochromes *a*, *b* and *c* rapidly became visible at 603 $m\mu$, 562-563 $m\mu$ and 551-552 $m\mu$ respectively. The α -bands of cytochromes *a* and *c* appeared almost simultaneously closely followed by the α -band of cytochrome *b*. The complete reduction took about 15 sec. In the cell containing bicarbonate the order of reduction of the cytochromes was the same, but the rate was slower with complete reduction being attained in about 30 sec. In both cases the cytochromes were reoxidized on shaking in air. The addition of a further 0.4 ml. of carbon dioxide-bicarbonate mixture increased the time for complete reduction to 45 sec., whereas the addition of a corresponding amount of sodium chloride to the control was without effect. Again in both cases the cytochromes were reoxidized on shaking with air.

The suggestion from these observations that the inhibition by bicarbonate or carbon dioxide occurred at a point below cytochrome *b* in the respiratory chain was consistent with the following qualitative observations. In an evacuated Thunberg tube the reduction of methylene blue by the

mitochondria, with succinate as substrate, was markedly inhibited in the presence of carbon dioxide-bicarbonate mixture at a final concentration corresponding to 30% of carbon dioxide. No change was detectable with the microspectroscope in the spectrum of reduced cytochrome *c* in the presence of a high concentration of the carbon dioxide-bicarbonate mixture. There was no detectable change in the appearance of the α -band of cytochrome *a* when, in an evacuated Thunberg tube, sodium bicarbonate was tipped from the side arm into a reaction mixture containing mitochondria and succinate, to yield a final concentration of 0.6M.

Spectrophotometric measurements

The effects of various concentrations of bicarbonate-carbon dioxide buffer on the succinic- and DPNH-cytochrome *c* reductase systems and on cytochrome *c* oxidase are shown in Figs. 1-3. The activity of succinic-cytochrome *c* reductase fell off rapidly with increasing carbon dioxide concentration above 5%, as little as 12% of carbon dioxide causing a 50% inhibition. DPNH-cytochrome *c* reductase was also inhibited by carbon dioxide concentrations above about 30% but the activity fell off less rapidly than that of succinic-cytochrome *c* reductase and appreciable inhibition occurred only at relatively high carbon dioxide concentrations. The carbon dioxide concentration required for 50% inhibition was about 60%. On the other hand, cytochrome *c* oxidase showed a marked increase in activity at carbon dioxide concentrations

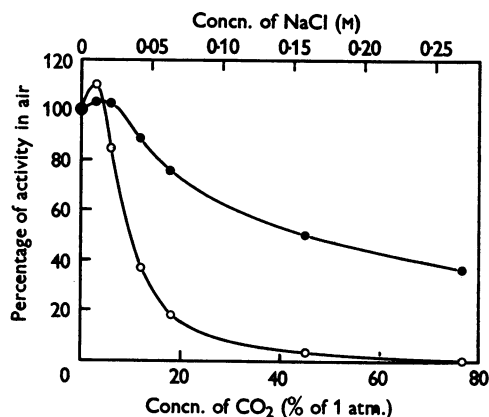


Fig. 1. Effect of CO_2 -bicarbonate mixtures (O) and of NaCl (●) on the succinic-cytochrome *c* reductase system. The reaction mixtures contained, in a final volume of 3 ml.: 0.67% of tris-HCl buffer, pH 7.4, 31 μ M-cytochrome *c*, 0.5 mM-KCN, pH 7.4, 20 mM-sodium succinate, enzyme and the appropriate quantity either of 0.316M- $NaHCO_3$, which had been equilibrated against $CO_2 + O_2$ (90:10) at 25°, or of 0.316M-NaCl. Temp. 25°.

around 20% but at higher concentrations the activity declined and above 60% of carbon dioxide the activity was markedly below that in air. The degree of stimulation observed in different preparations was rather variable, and in one case the maximum activity was double that in the absence of carbon dioxide.

In order to obtain further information about the types of inhibition involved with the cytochrome reductases the rates of reaction were measured at different concentrations of succinate or DPNH for a series of carbon dioxide concentrations. In Figs. 4

and 5 the results have been plotted according to the method of Lineweaver & Burk (1934) for the succinic- and DPNH-cytochrome *c* reductases respectively. It seems clear that the inhibition of succinic-cytochrome *c* reductase was essentially of the competitive type, whereas that of DPNH-cytochrome *c* reductase was non-competitive. However, the Lineweaver-Burk plots for the succinic system passed through a point which lay a little off the $1/v$ axis, which suggests that there may have been a small contribution from a non-

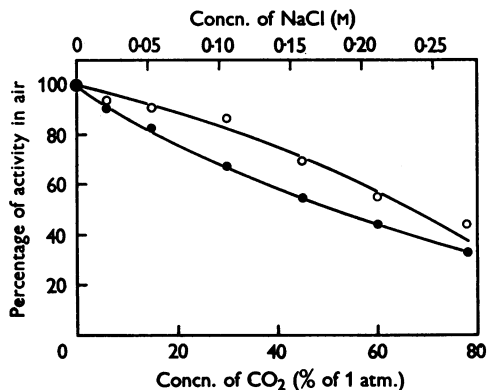


Fig. 2. Effect of CO₂-bicarbonate mixtures (O) and NaCl (●) on the DPNH-cytochrome *c* reductase system. The reaction mixtures were as given in Fig. 1 except that sodium succinate was replaced by 0.129 mM-DPNH. Temp. 25°.

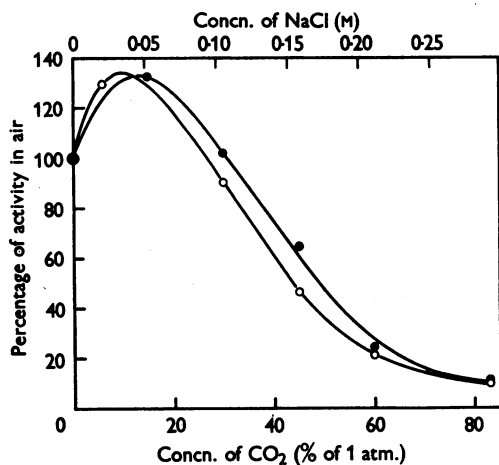


Fig. 3. Effect of CO₂-bicarbonate mixtures (O) and NaCl (●) on cytochrome oxidase. The reaction mixtures contained, in a final volume of 3 ml.: 0.67% of tris-HCl buffer, pH 7.4, 14.9 μM-reduced cytochrome *c* and the appropriate quantity of either CO₂-bicarbonate mixture or NaCl as in Fig. 1. Temp. 25°.

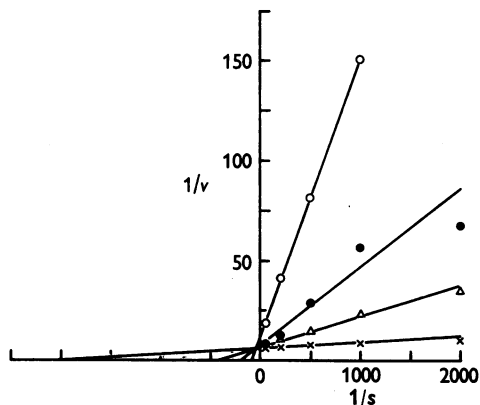


Fig. 4. Effect of succinate concentration on succinic-cytochrome *c* reductase at different concentrations of CO₂-bicarbonate buffer. v , reaction velocity (μmoles of cytochrome *c*/min./g. of fresh tissue); s , succinate concn. (M). O, 17.3% of CO₂; ●, 8.7% of CO₂; Δ, 4.3% of CO₂; ×, no CO₂. Other reaction conditions were as given in Fig. 1.

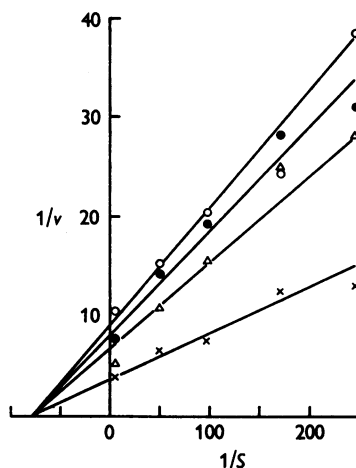


Fig. 5. Effect of DPNH concentration on DPNH-cytochrome *c* reductase at different concentrations of CO₂-bicarbonate buffer. v , reaction velocity (μmoles of cytochrome *c*/min./g. of fresh tissue); S , DPNH concn. (mM). O, 78% of CO₂; ●, 60.6% of CO₂; Δ, 43.4% of CO₂; ×, no CO₂. Other reaction conditions were as given in Fig. 2.

competitive type of inhibition (Dixon & Webb, 1958). This could have been due to a non-specific salt effect of the sodium bicarbonate since sodium chloride also gave some inhibition at low concentrations (Fig. 1).

The competitive nature of the inhibition of the succinic system indicates that the inhibition is reversible. The following observations suggest that the inhibitions of the other enzymic systems by high concentrations of carbon dioxide and bicarbonate are also reversible. Two samples of the original mitochondrial preparation were diluted tenfold, one by the addition of water, the other by the addition of carbon dioxide-bicarbonate buffer to give a final mixture in equilibrium with 80% of carbon dioxide. They were allowed to stand in the cold for 30 min. Cytochrome oxidase and DPNH-cytochrome *c* reductase activities were then assayed in reaction mixtures containing 0.1 ml. of the treated mitochondrial preparations in a final volume of 3 ml. If the combination of carbon dioxide or bicarbonate with the enzymes had been irreversible the activities in the preparations treated with carbon dioxide-bicarbonate would have been very markedly inhibited. In fact the activities of DPNH-cytochrome *c* reductase and of cytochrome *c* oxidase in the preparation treated with carbon dioxide-bicarbonate mixture were 99 and 114% respectively of those in the sample diluted with water.

Effects of salts other than bicarbonate

To test the specificity of the inhibitions by carbon dioxide-bicarbonate mixtures, the effects of salts other than sodium bicarbonate on the three enzymic systems were investigated. The effects of sodium chloride on the DPNH-cytochrome *c* reductase and on the cytochrome oxidase were clearly similar to those of sodium bicarbonate (Figs. 2, 3). Inhibitory effects were obtained also with other salts, e.g. the percentage inhibitions of the reductase by sodium bicarbonate, sodium chloride, potassium chloride and sodium sulphate (each 0.274M) and by 0.137M-sodium sulphate were 56, 67, 63, 78 and 59% respectively. In contrast the inhibition of the succinic-cytochrome *c* reductase by sodium chloride was markedly less than by bicarbonate (Fig. 1).

The comparisons of the effects of sodium chloride and sodium bicarbonate-carbon dioxide mixture shown in Figs. 1, 2 and 3 are for equivalent concentrations of sodium ion.

DISCUSSION

The inhibitions of the succinic and DPNH oxidase systems by carbon dioxide-bicarbonate mixtures appear to be of two types. The first kind of inhibition affected the succinic-cytochrome *c* reductase

system only, and it was appreciable at concentrations of carbon dioxide below 20%. The fact that the inhibition was competitive with respect to succinate and that sodium chloride had only a small effect on the system in the equivalent concentration range both indicate that this type of inhibition is a specific effect of the carbon dioxide mixture. The competitive nature of the inhibition also suggests that the actual component of the system affected was succinic dehydrogenase. This interpretation is consistent with the observations in the spectroscopy and Thunberg experiments which indicated that the inhibition occurs at some point below cytochrome *b* in the succinic-oxidase chain. They provided no evidence of an inhibition at any point in the chain between cytochrome *b* and cytochrome *a*. Whether the inhibition was due to carbon dioxide or bicarbonate or possibly to both components of the mixture is at present unknown.

The second kind of inhibition is a relatively non-specific effect of high salt concentration which affected all three enzyme systems studied. The similarity of the effects of sodium chloride and carbon dioxide-bicarbonate mixtures on the DPNH-cytochrome *c* reductase and cytochrome *c* oxidase systems suggests that the effects of the solutions containing high carbon dioxide concentrations on those systems was probably due not to carbon dioxide itself but to the high sodium bicarbonate concentration. It is not yet clear whether this was an effect due to the anion or the cation. The specific inhibition of the cytochrome oxidase of root mitochondria by bicarbonate observed by Miller & Evans (1956) was not observed under the different experimental conditions of the present work.

From the foregoing it appears that the marked inhibition of succinic oxidase in the intact mitochondria (Ranson *et al.* 1960) in bicarbonate mixtures in equilibrium with carbon dioxide concentrations above about 10% can be attributed very largely to inhibition of the succinic-dehydrogenase component. The further inference from the earlier experiments that pyruvic oxidase may be inhibited at still higher concentrations of carbon dioxide and bicarbonate may possibly relate to the inhibition of DPNH-cytochrome *c* reductase and cytochrome *c* oxidase by the bicarbonate.

To what extent all of these results are relevant to the effects observed when intact plant tissues are exposed to atmospheres containing high concentrations of carbon dioxide is at present uncertain. It seems probable, however, that the accumulation of succinate noted in a variety of plant tissues after storage in atmospheres containing 10-20% of carbon dioxide (Ranson, 1953; Hulme, 1956; Ulrich & Landry, 1956) is related to an inhibition of succinic dehydrogenase in the tissues. The observation that the inhibition of this enzyme by carbon

dioxide is of a competitive nature would account for such variation as occurs in the level of carbon dioxide required to bring about succinate accumulation in different plant tissues.

Whether or not very high concentrations of carbon dioxide in the atmosphere around a plant tissue could in fact result in a sufficiently high bicarbonate concentration in the cytoplasm to bring about the inhibition of DPNH-cytochrome *c* reductase and cytochrome *c* oxidase is not known. If this did occur the combined inhibition of these enzymes together with that of the succinic dehydrogenase would go a long way towards explaining the onset of carbon dioxide zymasis (Thomas, 1925) in atmospheres containing carbon dioxide in concentrations approaching 50% and abundant oxygen.

SUMMARY

1. The rates of three reactions concerned in the oxidation of succinate and reduced diphosphopyridine nucleotide by *Ricinus* mitochondria were determined in reaction mixtures in equilibrium with various concentrations of carbon dioxide at pH 7.4 and 25°.

2. Succinic-cytochrome *c* reductase was markedly inhibited by relatively low concentrations of carbon dioxide-bicarbonate but only slightly by corresponding concentrations of sodium chloride. The inhibition was competitive with respect to succinate concentration. For this and other reasons it is inferred that the carbon dioxide-sensitive component of the reductase is succinic dehydrogenase.

3. Reduced diphosphopyridine nucleotide-cytochrome *c* reductase was inhibited by high concentrations of sodium bicarbonate and other salts.

4. Cytochrome *c* oxidase was stimulated by low

concentrations and inhibited by high concentrations of sodium bicarbonate and other salts.

5. The results have been discussed in relation to the observed effects of carbon dioxide on the oxidation of succinate and other acids by intact mitochondria and the effects of carbon dioxide in the environment around intact plant tissues.

The work described in this paper was started and carried out for the most part in the Department of Botany, King's College, Newcastle. The authors wish to express their thanks to Professor M. Thomas, F.R.S., for his continued interest and assistance. Several experiments were also carried out with apparatus financed by the D.S.I.R. and University of London, Central Research Fund. We are also grateful for a quantity of *Ricinus* seeds provided by the Baker Castor Oil Co., San Diego, Calif., U.S.A. One of us (D.S.B.) is indebted to the Agricultural Research Council for a research studentship held while part of the work was in progress.

REFERENCES

- Bendall, D. S., Ranson, S. L. & Walker, D. A. (1958). *Nature, Lond.*, **181**, 133.
 Dixon, M. & Webb, E. C. (1958). *Enzymes*. London: Longmans Green and Co.
 Hulme, A. C. (1956). *Nature, Lond.*, **178**, 218.
 Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
 Margoliash, E. (1954). *Biochem. J.* **56**, 259.
 Miller, G. W. & Evans, H. J. (1956). *Nature, Lond.*, **178**, 974.
 Ranson, S. L. (1953). *Nature, Lond.*, **172**, 252.
 Ranson, S. L., Walker, D. A. & Clarke, I. D. (1957). *Biochem. J.* **66**, 57 p.
 Ranson, S. L., Walker, D. A. & Clarke, I. D. (1960). *Biochem. J.* **76**, 216.
 Thomas, M. (1925). *Biochem. J.* **19**, 927.
 Ulrich, R. & Landry, J. (1956). *C.R. Acad. Sci., Paris*, **242**, 2757.
 Walker, D. A. & Brown, J. M. A. (1956). *Biochem. J.* **67**, 79.

Biochem. J. (1960) **76**, 225

The Measurement of the Diffusion of Oxygen through Respiring Tissue

By I. S. LONGMUIR AND ANN BOURKE

Department of Biochemistry, Institute of Diseases of the Chest, London, S.W. 3

(Received 8 January 1960)

The diffusion coefficient of oxygen through non-respiring tissue has been measured by Krogh (1919) The figure he obtained was a little lower than that through water. Similar values have been obtained by other workers (Kirk & Johnsen, 1951). In general the diffusion coefficient of oxygen through tissue appeared to be equal to that through water multiplied by the fraction of the tissue composed of

water. It seemed that the transport of oxygen through tissues consisted of simple diffusion through the aqueous part uninfluenced by the solid part. That this is not universally true was shown by Longmuir & Roughton (1952), who found that the diffusion coefficient of nitrogen and carbon monoxide through haemoglobin solutions of the concentration found in red cells was about one-