Effects of Carbon Dioxide–Bicarbonate Mixtures on Oxidative Phosphorylation by Cauliflower Mitochondria

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1. Carbon dioxide–bicarbonate mixtures markedly inhibited oxidation and phosphorylation rates of mitochondria prepared from cauliflower. Inhibition occurred with succinate, malate, citrate, isocitrate and NADH as substrates.
2. Indophenol-reductase systems with malate, succinate, isocitrate and NADH as substrates were inhibited by 5% and 15% carbon dioxide. Cytochrome *c* oxidase was not inhibited by 15% carbon dioxide.

Chlorosis and diminished growth may be manifested by plants cultured in a medium containing high concentrations of bicarbonate or carbon dioxide (Porter & Thorne, 1955). The way in which carbon dioxide–bicarbonate mixtures cause these disturbances is unknown. The present investigation was initiated in an effort to solve that problem.

Bendall, Ranson & Walker (1958) observed that the succinate-oxidase system in higher plants was sensitive to carbon dioxide–bicarbonate mixtures. Components of the respiratory chain were studied and it was concluded that the succinate–cytochrome *c*-reductase component of the system was the most markedly affected by carbon dioxide–bicarbonate mixtures (Bendall, Ranson & Walker, 1960; Ranson, Walker & Clarke, 1960). Inhibition occurred at bicarbonate concentrations about 50 mM in equilibrium with 10–15% carbon dioxide at pH 7.4. The inhibition was competitive with respect to succinate concentration. Carbon dioxide–bicarbonate mixtures inhibited NADH–cytochrome *c* reductase and cytochrome *c* oxidase at higher concentrations.

Miller & Chen (1961) found that both oxidation and phosphorylation rates of mitochondria separated from etiolated pea leaves were inhibited by 10% carbon dioxide in equilibrium with 12.4 mM-sodium hydrogen carbonate.

In the present investigation, mitochondria isolated from cauliflower were tested to determine whether carbon dioxide–bicarbonate mixtures had similar effects on both the phosphate-esterification process and the electron-transfer system. Experiments were initiated to determine the site or sites in the electron-transport system where inhibition occurred. The experimental design failed to differentiate between the effects of HCO_3^- ions and dissolved carbon dioxide; consequently, the effects on

electron transfer and phosphate esterification were expressed in terms of carbon dioxide–bicarbonate mixtures.

EXPERIMENTAL

Preparation of mitochondrial fractions. A method similar to that outlined by Wedding & Black (1962) was used. With the aid of a TenBroeck homogenizer, the mitochondrial pellet from cauliflower (*Brassica oleraceae* L.) was suspended in 50 ml. of 0.6 M-sucrose in 50 mM-tris buffer (adjusted to pH 7.2 with HCl). Resedimentation was achieved at 15000g for 25 min. The resuspending and resedimentation procedures were repeated once. The particles finally were suspended in 5 ml. of 0.4 M-sucrose in 50 mM-tris–HCl buffer, pH 7.0. The preparation contained 5–8 mg. of protein/ml. of mitochondrial suspension and was used for studies of oxidative phosphorylation and dehydrogenase activities.

Measurement of oxidative phosphorylation. The O_2 consumption was measured with a recording oxygen cathode (Oxygraph; Gilson Medical Electronics, Middleton, Wis., U.S.A.). The change in concentration of O_2 in the reaction solution was detected as the change in cathode-limiting current and recorded automatically. Each reaction cell used had 3 ml. capacity and two narrow necks. One neck was used for inserting the electrode and the other for adding reaction components. The reaction mixture was initially stirred and a rapidly oscillating cathode was used throughout the assay period. The concentration of O_2 in the air-saturated iso-osmotic medium was 250 μM at 25° (Chance & Smith, 1958).

For the measurement of oxidation of the substrate, the reaction mixture contained the following constituents: sucrose (0.28 M), MgCl_2 (4.66 mM), KCl (9.32 mM), EDTA (0.233 mM), KH_2PO_4 – K_2HPO_4 buffer (4.66 mM), tris–HCl buffer, pH 7.25 (9.32 mM), glucose (18.6 mM), ADP (0.186 mM), hexokinase [Sigma type II, 150000 Kunitz & MacDonald (1946) units/g.] (0.052%), ATP (0.7 mM) and NAD^+ (0.186 mM) (omitted when succinate or NADH was substrate). A CO_2 –bicarbonate mixture was added to adjust the pH to 7.25. NaCl was added to all solutions at

concentrations sufficient to compensate for the additions of NaHCO_3 to certain treatments and to give equimolar Na^+ concentrations. The mitochondrial preparation was added to start the reaction. Malate, succinate, citrate and isocitrate were added to give 3.72 mM final concentration; NADH was added to give 0.932 mM final concentration. The assay medium contained mitochondria at a concentration of 1.0–1.5 mg. of protein/3 ml. Preliminary results showed that cytochrome *c* had no effect on the oxidation rate; consequently it was not added to the reaction mixture. The temperature was maintained at 25° throughout the assay period.

For the determination of phosphorylation, inorganic [^{32}P]phosphate (1×10^6 – 4×10^6 counts/min./ml.) was included with the components listed for oxidation determinations. A 0.5 ml. sample of the reaction mixture (assay mixtures were incubated separately but under identical conditions for oxidation and phosphorylation determinations) was withdrawn soon after the mitochondrial suspension had been added, and subsequent 0.5 ml. samples were withdrawn at 5 min. intervals for 20 min.

Esterified [^{32}P]phosphate in each sample was analysed according to the reversed-phase chromatographic method developed by Hagihara & Lardy (1960). A 0.5 ml. sample was added to 1 ml. of reagent A [0.9 N- HClO_4 –0.6 M- Na_2SO_4 (final concentrations)]. Then 9.5 ml. of reagent B was added to the mixtures [reagent B contained 0.5 vol. of 4% (w/v) $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$, 0.4 vol. of methanol, 0.012 vol. of hexanol and about 0.15 vol. of water to make a final volume of 1.0, and was prepared just before use]. After standing for a few minutes, the solutions were poured into silicone-treated Celite columns, which were mounted on test tubes. The columns had flow rates of 1–3 ml./hr.

A 0.4 ml. sample of effluent from each column was dried in glass planchets and radioactivity was measured with a Geiger-Müller counter. Counts were corrected for background.

Assay of enzyme activity. For the measurement of indophenol-reductase activity, all assays were conducted with a Beckman model DU spectrophotometer in cuvettes of 1 cm. light-path at room temperature (25°). Activity was measured by following the decrease in extinction at 620 m μ after the reduction of 2,6-dichlorophenol-indophenol. The initial reading was taken 15 sec. after adding the mitochondria, and subsequent readings were recorded every 30 sec. for 4 min.

Constituents in the assay medium were the same as listed for the oxidation experiments plus KCN (3.4 mM) and 2,6-dichlorophenol-indophenol (2.7 μM).

In the absence of added substrate, reduction of indophenol was negligible during the assay period, indicating the absence of endogenous substrates or reducing substances. The rate of reduction of indophenol was proportional to enzyme concentration over the range used in these studies. No activity was obtained with a boiled extract preparation.

Cytochrome *c*-oxidase activity was determined by following the decrease in extinction at 550 m μ . The initial reading was taken 15 sec. after the addition of the mitochondrial preparation and subsequent readings were taken every 30 sec. during a 5 min. period. The assay medium contained the following constituents: sucrose (0.32 M), MgCl_2 (5.2 mM), KCl (10.4 mM), EDTA (0.26 mM), glucose (20.8 mM), phosphate buffer (5.2 mM), tris-HCl buffer, pH 7.25 (10.4 mM),

ADP (0.35 mM), reduced cytochrome *c* (16 μM) and mitochondria containing 50–75 μg . of protein. Reduced cytochrome *c* was prepared by adding dithionite and aerating for 5 min. with a fine stream of air.

Regulation of carbon dioxide concentration. Assay solutions containing NaHCO_3 were adjusted to pH 7.25 at 25° by equilibration with gas mixtures of 5% and 15% CO_2 in air (Matheson Co., Newark, Calif., U.S.A.). At pH 7.25, the final bicarbonate concentrations in the assay mediums were 29 mM and 9.4 mM in the presence of 15% and 5% CO_2 respectively. NaCl was added to adjust the total Na^+ concentration to 29 mM in all treatments. Gas was bubbled through all assay media (minus mitochondria) for 15 min. The pH value was checked at this time and at the end of the assay period to assure constant experimental conditions.

RESULTS

Effects of ADP and mitochondrial protein concentration on oxidation rates. Under the conditions of the assay with succinate as substrate, the oxidation rate was proportional to the concentration of mitochondrial protein (Fig. 1). Oxygen uptake at 25° was about 60 μg .atoms/min. with 1 mg. of protein and about 165 μg .atoms/min. with 3 mg. of protein.

When succinate was added to the assay medium in the absence of ADP, oxygen consumption in the

Fig. 1. Rate of O_2 uptake by cauliflower mitochondria with respect to mitochondrial protein concentration. In a final volume of 2.7 ml., the reaction mixtures contained: sucrose (0.28 M), MgCl_2 (4.66 mM), KCl (9.32 mM), EDTA (0.233 mM), KH_2PO_4 – K_2HPO_4 buffer (4.66 mM), tris-HCl buffer, pH 7.25 (9.32 mM), glucose (18.6 mM), ADP (0.186 mM), hexokinase (0.052%), ATP (0.7 mM) and succinate (3.72 mM).

2.7 ml. assay mixture was 62 $\mu\text{g}.$ atoms/min./mg. of protein. The oxygen uptake was accelerated to 95 $\mu\text{g}.$ atoms/min./mg. of protein with the addition of ADP (Fig. 2). This increase in respiration continued until all the oxygen in the reaction mixture was consumed.

Effects of carbon dioxide-bicarbonate mixtures on the rates of oxidation and phosphorylation. Preliminary studies on the effects of different carbon dioxide-bicarbonate concentrations on the oxidation of Krebs-cycle acids showed that 9.7 mm-bicarbonate (5% carbon dioxide) had little effect on the oxidation rate, 58 mm-bicarbonate (30% carbon dioxide) inhibited the oxidation rate almost completely and 29 mm-bicarbonate (15% carbon dioxide)

partially inhibited oxidation. Therefore 29 mm-bicarbonate was used in the experiments involving oxidative phosphorylation.

The rates of oxidation and phosphorylation by cauliflower mitochondria, when NADH and acids of the Krebs cycle were used as substrates, are shown in Table 1. These values are based on three or more measurements for each substrate, with different mitochondrial preparations used for every measurement. The rates of oxidation and phosphorylation with all substrates were higher in the control than in the carbon dioxide-bicarbonate treatments.

When succinate was used as the substrate in the presence of 29 mm-bicarbonate and 15% carbon dioxide, the rates of phosphorylation and oxidation were inhibited by 55 and 39% respectively. When citrate was used as the substrate under comparable conditions, the rates of phosphorylation and oxidation were inhibited by 35 and 26% respectively. With malate as substrate, the phosphorylation rate was inhibited by 73% and the oxidation rate by 30%.

The rate of oxidation of NADH was inhibited by only 16% by the carbon dioxide-bicarbonate treatment, but the phosphorylation rate was inhibited by 40%. Isocitrate was not an effective substrate with the mitochondrial preparation used.

Effect of carbon dioxide-bicarbonate mixtures on Krebs-cycle acid-indophenol-reductase systems. The effects of carbon dioxide-bicarbonate mixtures on indophenol-reductase activity with various substrates are shown in Table 2.

Regardless of substrate, enzyme activities showed about 17% inhibition with the 5% carbon dioxide treatment and 30% inhibition with the 15% carbon dioxide treatment. As with the Oxygraph experiments, much more oxidation was noted when NADH was the substrate.

Cytochrome c-oxidase activity was studied with the oxygen electrode. No differences were found in

Fig. 2. Rate of O₂ uptake by mitochondria in relation to the addition of ADP (at B) with succinate (added at A) as substrate. The reaction mixtures were as given in Fig. 1, except that 1.5 mg. of protein was added as mitochondria.

Table 1. *Effect of carbon dioxide-bicarbonate mixtures on oxidative phosphorylation with Krebs-cycle acids and NADH as substrates*

Experimental details are given in the text.

Substrate	Control (air only)		15% CO ₂ in air			
	P _i uptake	O ₂ uptake	P _i uptake		O ₂ uptake	
	(mμmoles/min./mg. of protein)	(mμg.atoms/min./mg. of protein)	(mμmoles/min./mg. of protein)	(% of control)	(mμg.atoms/min./mg. of protein)	(% of control)
Succinate	75.4	87.8	33.8	45	54.0	61
Malate	62.9	56.0	16.7	27	39.2	70
Citrate	16.8	38.0	11.0	65	28.0	74
Isocitrate	1.5	13.9	0.7	47	7.8	56
NADH	71.2	101.0	42.7	60	85.1	80

Table 2. *Effect of carbon dioxide-bicarbonate mixtures on Krebs-cycle acid-indophenol-reductase activity at pH 7.25*

Experimental details are given in the text. All substrates used were at 3.72 mM.

Substrate	Indophenol-reductase activity				
	Control (air only)	5% CO ₂ in air		15% CO ₂ in air	
	(mμmoles/ min./mg. of protein)	(mμmoles/ min./mg. of protein)	(% of control)	(mμmoles/ min./mg. of protein)	(% of control)
Succinate	1.92	1.61	84	1.34	70
Malate	4.00	3.30	83	2.88	72
NADH	13.50	11.08	82	9.78	73
Citrate	1.61	1.37	83	1.14	71
Isocitrate	1.39	1.06	82	0.97	70

Fig. 3. Lineweaver-Burk plot of the inhibition of indophenol reductase by CO₂-bicarbonate mixtures with succinate as substrate. The reaction mixtures were as given in Fig. 1, except for the concentrations of substrate and CO₂-bicarbonate indicated. O, Control with 29 mM-NaCl; Δ, 5% CO₂-9.4 mM-NaHCO₃; ●, 15% CO₂-29 mM-NaHCO₃. Fig. 4. Lineweaver-Burk plot of the inhibition of indophenol reductase by CO₂-bicarbonate mixtures with malate as substrate. The reaction mixtures were as given in Fig. 1, except for the concentrations of substrate and CO₂-bicarbonate indicated. O, Control with 29 mM-NaCl; Δ, 5% CO₂-9.4 mM-NaHCO₃; ●, 15% CO₂-29 mM-NaHCO₃.

the rate of oxygen uptake between the control and the 15% carbon dioxide-bicarbonate treatments. The rate of oxidation was 39 μg.atoms/min./mg. of protein. The phosphorylation rate was too low to allow accurate measurements.

Studies on inhibition. To learn what types of inhibition were involved in the indophenol-reductase activity in the presence of carbon dioxide-bicarbonate mixtures, rates of activity at several carbon dioxide-bicarbonate concentrations at various substrate concentrations were measured. A reciprocal plot according to the method of Lineweaver & Burk (1934) is illustrated in Fig. 3 with succinate as substrate and in Fig. 4 with malate as substrate. For both substrates inhibition seems to be enhanced at low substrate concentrations and suppressed at high substrate concentrations (competitive). The threshold point at which competitive

changes to substrate-promoted inhibition is apparently at a higher substrate concentration when the carbon dioxide-bicarbonate concentration is relatively low.

DISCUSSION

In this series of experiments, the slight increase in mitochondrial respiration rates after ADP was added indicated a loose coupling of the respiratory system to the phosphorylating system. The carbon dioxide-bicarbonate treatments significantly decreased oxidation and phosphorylation rates with all Krebs-cycle acid substrates used and with NADH. These results indicate that the respiratory chain is directly inhibited by carbon dioxide-bicarbonate mixtures.

When reduced cytochrome *c* was used as substrate, no difference was found between oxidation

rates after control and carbon dioxide-bicarbonate treatments. Cytochrome *c* oxidase was not sensitive to carbon dioxide-bicarbonate mixtures at low concentrations. This agrees with the results of Bendall *et al.* (1960). These workers found NADH-cytochrome *c* reductase from *Ricinus* mitochondria to be insensitive to carbon dioxide except at high concentrations. The inhibition at high concentrations was considered non-competitive with respect to NADH, and was concluded to be attributable to the high bicarbonate concentration. In contrast, they found that the reduction of cytochrome *c* by the succinate system was strongly inhibited by concentrations of carbon dioxide as low as 17% and this inhibition was considered to be competitive with respect to succinate.

Our results show that the oxidation and phosphorylation with four Krebs-cycle acids and NADH were all inhibited similarly by carbon dioxide-bicarbonate mixtures. Bendall *et al.* (1960) found a much greater inhibition of succinate oxidase and a smaller inhibition of NADH oxidase than we observed with comparable carbon dioxide-bicarbonate mixtures. The experiments, however, are not entirely comparable since preparation of mitochondria (non-iso-osmotic versus iso-osmotic) and assay methods were different.

Spectrophotometric determinations of indophenol-reductase activities indicated similar degrees of inhibition when Krebs-cycle acids or NADH were used as substrates. The dye was reduced by electron transfer from NADH or succinate, and thus the inhibitory site or sites would be between the substrate level and cytochrome *c*. Enzyme activities were much lower with 2,6-dichlorophenol-indophenol than with oxygen as the electron acceptor. The difference in activity might be accounted for if permeability of the mitochondria to the dye were limited.

The inhibition noted with carbon dioxide-bicarbonate mixtures may have been attributable to pH changes. No detectable pH difference was noted in the assay media of the various carbon dioxide-bicarbonate treatments. However, though carbon dioxide diffuses freely through membranes, the cell membrane is almost impermeable to HCO₃⁻ ions (Rabinowitch, 1945). Diffusion of carbon dioxide across the mitochondrial membrane could change the internal pH and affect oxidation rates.

The suppression of inhibition of indophenol reductase by carbon dioxide-bicarbonate mixtures at high substrate concentrations and the substrate-promoting inhibition at low concentrations suggests the possibility of at least two different receptor sites on the enzyme, each of which shows contrasting degrees of affinity for carbon dioxide-bicarbonate and the substrate. The complexity of the mitochondrial system, however, presents the possibility

that more than one enzyme with similar catalytic properties may be present and the degree of inhibition may vary with the different enzymes.

Ranson *et al.* (1960) conducted experiments with ¹⁴C-labelled substrates and followed the oxidation of these substrates in the presence of carbon dioxide-bicarbonate mixtures. The production of malate from succinate oxidation was diminished by about 40% in 10% carbon dioxide, and by more than 30% in 5% carbon dioxide. When fumarate was used as the substrate, carbon dioxide had no effect on malate synthesis. When ¹⁴C-labelled pyruvate was used as substrate in the presence of 30% carbon dioxide, the amounts of citrate and isocitrate being formed were decreased by 80% in 30 min. Some succinate was found, but it did not accumulate as one would expect if succinate oxidase were the only carbon dioxide-sensitive site. No malate was formed under these conditions. Ranson *et al.* (1960) therefore concluded that, in addition to succinate oxidase being affected, some enzyme involved in the transformation of pyruvate into citrate was carbon dioxide-sensitive. From our results it would seem that dehydrogenases in general are carbon dioxide-bicarbonate-sensitive. Inhibition of pyruvate dehydrogenase as well as of other dehydrogenases in the Krebs cycle would therefore be expected. The results obtained by Ranson *et al.* (1960) with ¹⁴C-labelled substrates would support the hypothesis of general sensitivity of all dehydrogenases to carbon dioxide-bicarbonate mixtures, but one would have to conduct experiments with other ¹⁴C-labelled Krebs-cycle intermediates before the theory could be proved.

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