Effects of Carbon Dioxide on Mitochondrial Enzymes from *Ricinus*

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Many aspects of the growth and development of plants and of their metabolism are known to be influenced by the carbon dioxide concentration in the surrounding atmosphere (see, for example Crocker, 1948; Harrison, 1953). Notwithstanding the number and variety of these observations, very little is yet known about the effects of carbon dioxide on specific enzyme systems in plants. From his discovery that exposure of plant organs to aerobic atmospheres containing high carbon dioxide concentrations resulted in the accumulation of ethanol and acetaldehyde, Thomas (1925) inferred that some oxidative process was inhibited. However, though carbon dioxide-zymasis (for definition see Thomas, 1925) is induced in many tissues by carbon dioxide in concentrations above about 50 %, it has not been reported in concentrations much below this value (Foster, 1940; Smith, 1957). Walker & Brown (1957) found that the activity of phosphoenolpyruvic carboxylase was decreased by about 30 % in the presence of 10 % of carbon dioxide and that inhibition of the enzyme increased with carbon dioxide concentration until in about 70 % of carbon dioxide the inhibition was virtually complete. The inhibition was shown to be of a non-competitive type and to our knowledge these observations provided the first direct demonstration of the inhibition of an enzyme by carbon dioxide in high concentration.

Succinic acid is known to accumulate in certain plant tissues when they are stored in atmospheres containing 20 % or in some cases 10 % of carbon dioxide (Ranson, 1953; Hulme, 1956; Ulrich & Landry, 1956). The experiments described in this paper were therefore primarily directed towards the investigation of the effects of carbon dioxide concentration on some enzymes involved in the production and consumption of succinate in plant tissues. A convenient source of these enzymes was a particulate preparation from the endosperm of *Ricinus* seedlings, isolated according to the method of Beevers & Walker (1956) and shown by them to exhibit a high tricarboxylic acid-cycle activity.

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The range of carbon dioxide concentrations studied was 0.05–90 %. Whereas concentrations in the upper half of this range may not occur in cells under natural conditions, concentrations in the lower half of the range may not be uncommon. For example, in a well-aerated soil the average carbon dioxide concentration is probably less than 1 %, but in the area around a well-developed root system a concentration of 9 % has been recorded (Russell & Appleyard, 1915), and Leonard (1945) has suggested that the concentration at the surfaces of a root system might on occasions rise to several times this value. In bulky plant organs, e.g. fruits, tubers and stems of trees, where gaseous exchange is impeded, the carbon dioxide concentration is again often very much higher than that of the surrounding atmosphere. The concentrations recorded for the fruits of apple and pear are variable and depend on many factors, including temperature, but average concentrations of 5–10 % within a fruit are common and concentrations nearer 30 % have been recorded (Magness, 1920). Since in such organs the concentration at the centre is presumably higher than that near the surface, carbon dioxide concentrations around 20 % may not be uncommon in the inner zones of many bulky tissues.

It has been suggested that some of the observed effects of high carbon dioxide concentrations in the atmosphere around plant tissues may result from increased acidity within the cells as a result of the solution of carbon dioxide in the sap (see, for example, Small, 1954). Thornton (1933), however, found that saps expressed from a variety of tissues, after storage in high carbon dioxide concentrations, were invariably at a higher pH than saps expressed from similar tissues after storage in air. The increase in pH was ascribed to secondary effects of the carbon dioxide treatment. It is, however, doubtful whether such observations on the overall pH within tissues or cells throw any light on the direct effect of carbon dioxide on the pH within the cytoplasm of cells. The experiments described in this paper were confined therefore to conditions under which the carbon dioxide concentration was varied at a single constant pH value.

A preliminary account of some of this work has already appeared (Ranson, Walker & Clarke, 1957).

MATERIALS AND METHODS

Preparation of mitochondrial fractions. The method was essentially that described by Beevers & Walker (1956). Castor beans (*Ricinus communis* var. Cimarron) were germinated for 4 days in moist, coarse-grade horticultural Vermiculite (heat-expanded mica) at 30° in the dark. The endosperm tissue was then separated and ground in a medium containing 0.5 M-sucrose, 6 mM-MgCl₂ and 0.1 M-NaH₂PO₄, and adjusted to pH 7.4 with KOH. The mitochondrial fraction separated by differential centrifuging was washed once and finally resuspended in the grinding medium. The volume of the final suspension was such that 1 ml. contained particles from 10–15 g. of endosperm.

Substrates and cofactors. Stock solutions (0.2 M) of each of the substrate acids were prepared in deionized water and adjusted to pH 7.4 with NaOH. [1-¹⁴C]- and [2:3-¹⁴C]-Succinic acid, [1-¹⁴C]- and [2:3-¹⁴C]-fumaric acid and sodium [3-¹⁴C]pyruvate, purchased from The Radiochemical Centre, Amersham, were diluted with their inactive analogues, dissolved immediately before use and the solutions were adjusted to pH 7.4. Unless otherwise stated the final concentration of each acid in reaction mixtures was 0.01 M.

Diphosphopyridine nucleotide (DPN), adenosine triphosphate (ATP), coenzyme A (CoA) and cocarboxylase were purchased from Sigma Chemical Co., St Louis, Mo., U.S.A. These were dissolved in deionized water, adjusted to pH 7.4 and added as required to the reaction mixtures to provide 1 mg. of DPN, 1 mg. of ATP, 0.1 mg. of CoA and 0.5 mg. of cocarboxylase/ml. of mitochondrial preparation used. The final concentrations (mM) of these cofactors in reaction mixtures were approximately 0.4, 0.6, 0.04 and 0.35 respectively.

Regulation of carbon dioxide concentration. Aqueous solutions of NaHCO₃ were prepared at molar concentrations calculated on the basis of the Henderson-Hasselbalch equation (see Umbreit, Burris & Stauffer, 1949) to adjust to pH 7.4 at 25° when equilibrated with gas mixtures containing 5, 10, 20 and 30% of CO₂ in air and 50, 70 and 90% of CO₂ in O₂. The bicarbonate concentrations were adjusted to allow for dilution on addition to the final reaction mixtures. Gas was bubbled through the solutions for several hours before use.

Activity measurements. Uptakes of oxygen of the particulate preparations in the presence of added substrate acids and cofactors were measured manometrically as described by Beevers & Walker (1956).

Analysis of reaction products. Substrates, cofactors and CO₂-bicarbonate buffer were mixed in either 10 ml. test tubes or 30 ml. flasks fitted with inlets and outlets and the mixtures equilibrated at 25° against gas streams containing the appropriate CO₂ concentration for about 30 min. The particulate preparation was then added and the gas stream continued. In most of the experiments described below, 1 ml. or 2 ml. samples were removed from the final reaction mixture after appropriate time intervals and rapidly added to 20 ml. of boiling 70% ethanol. The mixture was then concentrated to about 1 ml., adjusted to pH 1 by the addition of HCl, thoroughly stirred into about 15 g. of anhydrous Na₂SO₄ and transferred quantitatively to a Soxhlet thimble. Organic acids were extracted with anhydrous ether in the Soxhlet apparatus for 36–48 hr. The acids were then transferred to a measured amount of water and

analysed by chromatography in one or two dimensions on Whatman no. 1 paper with *tert*-amyl alcohol-formic acid-water (3:1:3, by vol.) and propanol-ammonia (6:4, v/v) as developing solvents (see, for example, Ranson, 1955).

In some of the later experiments with ¹⁴C-labelled substrates at a higher specific activity than those used in the earlier experiments, small amounts (5–20 μl.) of the reaction mixture were applied directly to the filter paper and dried rapidly with a hot-air stream to inactivate the enzymes. A mixture of known acids was then added in sufficient amount to allow positive identification by conventional spray techniques on the developed chromatogram of the actual components of the unknown mixtures.

Radioautographs were prepared from the developed chromatograms by placing the paper in contact with Ilfex (Ilford Ltd.) X-ray films for 7 days. Radioactive compounds, located on the paper with the aid of the developed film, were assayed for their ¹⁴C content either directly on the paper or after elution by means of a Geiger-counter tube fitted with a thin end-window. Compounds were eluted by placing active areas of the filter paper, cut into pieces about ½ in. square in stoppered tubes with 0.5–2.0 ml. of aq. mN-NH₃ and shaking for 1 hr. on a rotary shaker. Samples (0.3–0.5 ml.) of the ammoniacal extracts were dried on aluminium plates for ¹⁴C assay. Self-absorption corrections were applied in all ¹⁴C measurements.

RESULTS

The uptakes of oxygen of the various mitochondrial preparations, measured manometrically in the presence of added cofactors and Krebs-cycle acids, were broadly similar to those reported (Beevers & Walker, 1956). Chromatographic analysis of the acid products demonstrated the production of citric acid, *isocitric* acid, α -oxo-glutaric acid, succinic acid, fumaric acid and malic acid from their respective precursors in the acid cycle. Succinic acid was consumed with the production of fumaric acid and malic acids by mitochondrial preparations supplemented only with ATP, or without added cofactors.

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In these experiments the only cofactor added was ATP. Preliminary investigations in which the acids produced from succinate were measured by the spot-area method after paper chromatography suggested that the production of fumarate and malate was markedly inhibited in carbon dioxide-bicarbonate mixtures corresponding to gas concentrations above about 20%.

To allow more accurate quantitative measurements, [¹⁴C]succinate was used in subsequent experiments. In one of these (see legend to Fig. 1), the effects of carbon dioxide in concentrations between 0.05 (air) and 90% were compared. Samples were removed after time intervals up to 50 min. and inactivated with boiling 70% ethanol. In the ether-soluble constituents of these samples

the recovery of the ^{14}C supplied originally as succinate was uniformly better than 90%. The sum of the ^{14}C contents of well-defined radioactive components when counted directly on chromatograms varied on individual sheets between 75 and 105%. Since there was no apparent correlation between the percentage recovery and previous carbon dioxide treatment, the low recoveries are attributed to imperfections in technique rather than to loss of labile components during chromatography.

Visual examination of the radioautographs showed that in air containing up to 10% of carbon dioxide there was a steady depletion of succinate with a corresponding increase in malate and fumarate, whereas in 20% and higher concentrations of carbon dioxide consumption of succinate was slight. In addition to the labelled acids mentioned, other labelled components were present on chromatograms but none of these corresponded with the known positions of Krebs-cycle intermediates. Three of the unidentified compounds contained, in certain instances, considerable proportions of the total ^{14}C activity and these are referred to below as unknowns A, B and C. Their approximate co-ordinates relative to malic acid (100) were, for the alkaline solvent, A, 217; B, 225; C, 340; and, for the acid solvent, A, 70; B, 88; C, 61. As an example, counts made from a two-dimensional chromatogram of the products after

50 min. in air gave the following figures: succinate 900, malate 1600, fumarate 250, A 180, B 200 and C 100 counts/min., and there were other less-active components. After a 50 min. period in 70% of carbon dioxide most of the ^{14}C remained in succinate, but unknown compounds A, B and C were still detectably labelled.

Because of the production of these unidentified compounds from succinate, the activity appearing in malic acid appeared to be the safest measure of the oxidative consumption of succinate by way of succinic oxidase and fumarase, and the ^{14}C present in malic acid after various time intervals in gas atmospheres containing different carbon dioxide concentrations is recorded in Fig. 1. In 10% of carbon dioxide there was, overall, a 40% decrease in the rate of malate production. In higher concentrations malate production was more markedly retarded from the start and virtually stopped completely within about 25 min. The rate of malate production was faster initially in 5% of carbon dioxide than in air. A similar result was observed in one other experiment but its significance remains in doubt. [^{14}C]Fumarate was detected whenever labelled malate was produced but at amounts never greater than 25% of the malate. No label was detected in citrate in any gas concentration, though in parallel experiments in which the reaction mixtures contained DPN, CoA and cocarboxylase in addition to ATP, labelled citrate was readily detected after about 26 min. in air.

Since the major part of ^{14}C supplied to the mitochondrial preparation was accounted for on many of the chromatograms it appeared probable that malate produced was not converted into oxaloacetate or pyruvate to any marked extent, and that the malate accumulating represented substantially that produced from succinate. The conclusion drawn was that the oxidative consumption of succinate to yield malate was markedly inhibited in carbon dioxide concentrations of 20% and above. In the higher concentrations where malic acid production was retarded there was no accumulation of fumarate. It thus appeared probable that it was a component of the succinic oxidase which was sensitive to carbon dioxide.

Carbon dioxide concentration and fumarate consumption

When mitochondrial preparations were supplied with [$1:4\text{-}^{14}\text{C}_2$]fumarate in the presence of ATP only, in experiments otherwise similar to those described in the previous section, the fumarate was converted into malate at rates which were the same in high carbon dioxide concentrations as in air. Small amounts of ^{14}C were detectable again in several unidentified components but no other stable Krebs-cycle intermediate was labelled.

Fig. 1. [^{14}C]Malate produced from [^{14}C]succinate by mitochondria in equilibrium with atmospheres containing from 0.05 to 90% of CO_2 . Reaction mixtures contained 3 ml. of mitochondrial suspension, 6.9 mg. of ATP, 8 mg. of succinate containing $10\text{ }\mu\text{C}$ of [$1\text{-}^{14}\text{C}$]succinate, and CO_2 -bicarbonate mixture and water as required. The final volume was 6.9 ml., temperature 25° and pH 7.4.

Similar experiments in which the mitochondria were supplied with the four cofactors were carried out with [1:4-¹⁴C₂]- and [2:3-¹⁴C₂]-fumarate as substrates. In air the carboxyl-labelled acid yielded labelled citrate and isocitrate but no labelled succinate, whereas the fumaric acid labelled in the central carbon atoms yielded labelled citrate, isocitrate and, eventually, labelled succinate. These results are in accord with those which would be expected from present knowledge of the partial reactions of the Krebs cycle. Again the carbon dioxide concentration had no effect on the conversion of fumarate into malate.

Carbon dioxide concentration and pyruvate oxidation

Mitochondria supplemented with the four cofactors were supplied with [3-¹⁴C]pyruvate in reaction mixtures in equilibrium with air, and 30 and 70 % of carbon dioxide (see legend to Table 1). Unlabelled citric acid, malic acid, succinic acid and fumaric acid were included in the mixtures to serve as traps for their labelled analogues produced from the pyruvate. Samples were removed at intervals up to 90 min. and applied directly to origins on paper chromatograms. In developing the chromatograms the sheets were allowed to remain in the alkaline solvent for a sufficiently long time for residual pyruvate to run off the paper. They were then developed in the second direction in the acid solvent. The only labelled components present in other than trace amounts which could be detected on any sheets were citric acid, isocitric acid, malic acid, succinic acid, fumaric acid and alanine. The appearance of labelled alanine was attributed to the presence of an amino acid and of a transaminase capable of transferring an amino group from the acid to the labelled pyruvate. The presence of transaminases in the mitochondrial preparation had been demonstrated previously (D. A. Walker, unpublished work).

In air after 5 min. the ¹⁴C was present mainly in citric acid. With increase in time malic acid was

labelled, and after 90 min. contained more ¹⁴C than the citric (+ isocitric) acid. Succinate accumulated ¹⁴C slowly (see Table 1). In 30 % of carbon dioxide the incorporation of ¹⁴C from the pyruvate was markedly retarded and after 90 min. no measurable activity was present in either fumarate or malate. In 70 % of carbon dioxide the incorporation of ¹⁴C was very slight and the only compound (other than alanine) containing appreciable activity after 90 min. was citrate. Taking as a rough measure the total amount of ¹⁴C present after 90 min. in the Krebs-cycle acids detected on chromatograms, the relative amounts of pyruvate metabolized in air, 30 and 70 % of carbon dioxide were about 100, 24, and 5 respectively. The total activity present in the acids in air after 90 min. represented about 40 % of the ¹⁴C supplied originally. This fraction represents a minimum estimate of the pyruvate consumed.

The results described earlier indicate that the succinic oxidase would be markedly inhibited in both 30 and 70 % of carbon dioxide, and this inhibition might be expected to retard in time the utilization of pyruvate in Krebs-cycle reactions. The presence of activity in succinate after 90 min. in 30 % of carbon dioxide with no detectable trace in fumarate and malate points to the inhibition of the succinic oxidase in this experiment also. If, however, this oxidase were the only partial system inhibited, one might expect an accumulation of ¹⁴C in the succinate to an extent greater than that which occurred in air and, moreover, the accumulation of ¹⁴C in citric acid and other acids, e.g. α -oxoglutaric acid, which precede succinic acid in the cycle. Such accumulations were not observed. Moreover, the inhibition of pyruvate metabolism was greater in 70 % than in 30 % of carbon dioxide, which appears unlikely if succinic oxidase were the sole enzyme system affected. These results suggest therefore that in 30 and 70 % of carbon dioxide some enzyme involved in the production of citrate from pyruvate was markedly affected in addition to the succinic oxidase.

Table 1. *Incorporation of [¹⁴C]pyruvate into acids by mitochondrial preparations*

Values of ¹⁴C are in 10⁻³ × counts/min. Reaction mixtures contained in 1 ml. of mitochondrial suspension: 1 mg. each of DPN, ATP, cocarboxylase, 0.1 mg. of CoA, 1 mg. each of succinate, fumarate, L-malate, citrate, 1.6 mg. of pyruvate containing 30 μ C of [3-¹⁴C]pyruvate and CO₂-bicarbonate mixture and water as required. The final volume was 3 ml., temperature 25° and pH 7.4. No entry (—) in the table indicates activities less than 1.5 × 10³ counts/min.

	Air		30 % CO ₂		70 % CO ₂	
	30 min.	90 min.	30 min.	90 min.	30 min.	90 min.
Citric acid + isocitric acid	105	198	21	93	—	20
Succinic acid	—	18	—	15	—	—
Malic acid	15	225	—	—	—	—
Fumaric acid	2	24	—	—	—	—

DISCUSSION

In the previous section results have been described which demonstrate the sequential production of citric acid, isocitric acid, α -oxoglutaric acid, succinic acid, fumaric acid, and malic acid in mitochondrial preparations supplied with ATP, DPN, coenzyme A, cocarboxylase and suitable substrates. Moreover, in similar preparations with added cofactors and acid substrates labelled with ^{14}C the oxidative production of succinate from fumarate and of malate from pyruvate has been demonstrated. These results, together with previous manometric observations (Beevers & Walker, 1956, Walker & Beevers, 1956), are in keeping with the now well-established view that intact mitochondria can bring about the integrated reactions of the Krebs cycle. Particulate preparations from *Ricinus* are well suited to the study of these reactions in cell-free systems particularly since their activity is extremely high and their endogenous respiration negligible. The production in the particulate preparations of alanine from pyruvate would appear to confirm the presence of some transaminase activity. The production of the unknown compounds A, B and C (if in fact these compounds are not artifacts of the analytical technique) may indicate the presence of other enzymic activities. Most of the results described in this paper are, however, concerned with a comparison between the rates of several of the partial reactions in mixtures in equilibrium with air and with aerobic atmospheres containing various concentrations of carbon dioxide. For simplicity in this section we shall continue to refer results to the carbon dioxide concentration though it is possible that the operative factor is the bicarbonate ion in equilibrium with the carbon dioxide.

The main observation arising was that the conversion of succinate into malate was markedly affected by carbon dioxide in concentrations above about 10%. Since the conversion of fumarate into malate was apparently unaffected by such concentrations the conclusion drawn is that the succinic oxidase was the enzymic system inhibited. The results summarized in Fig. 1 indicate therefore that the oxidase was inhibited to the extent of about 40% by an atmosphere containing 10% of carbon dioxide and virtually completely inhibited by atmospheres containing 20% or more of carbon dioxide, under the particular conditions of this experiment. Work by Bendall, Ranson & Walker (1958, 1960) suggests, however, that the degree of inhibition may vary with the concentration of succinate in the system.

The experiments on the oxidative consumption of pyruvate were of a more exploratory nature. Nevertheless, they suggest that in 30% of carbon

dioxide, though succinate consumption was apparently completely inhibited, the incorporation of pyruvate into citrate continued for some time but at a much slower rate than in air. In 70% of carbon dioxide, the incorporation of pyruvate into citrate was very slow. One interpretation is that pyruvic oxidase or possibly condensing enzyme is sensitive to carbon dioxide but that complete inhibition occurred at a higher concentration than that required to inhibit succinic oxidase to the same degree.

Although at this stage attempts to relate the effects of atmospheres containing enhanced carbon dioxide concentrations on intact plant tissues to those observed with the particulate preparation must be cautious, it appears probable that the accumulation of succinate in plant organs stored in the presence of 10–20% of carbon dioxide can be attributed to an inhibition of succinic oxidase in those organs. Further, for plant tissues in which respiratory oxidations are mediated by mitochondrial enzymes the inhibition of succinic oxidase and possible other cycle enzymes by carbon dioxide may in part explain some of the reported effects of high concentrations of this gas on a variety of metabolic processes in plants.

An attempt to determine the site of the carbon dioxide inhibition in the succinic-oxidase system is reported elsewhere (Bendall *et al.* 1958, 1960).

SUMMARY

1. Reactions of the Krebs cycle in a mitochondrial preparation from *Ricinus* endosperm have been studied in air and in carbon dioxide concentrations up to 90%.

2. Succinic oxidase is markedly inhibited in carbon dioxide concentrations of 10% and above. At higher concentrations the conversion of pyruvate into cycle acids is also depressed, suggesting an inhibitory effect of carbon dioxide on pyruvic oxidase or condensing enzyme.

3. The results are discussed in relation to the effects of carbon dioxide on intact plant tissues under aerobic conditions.

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

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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