

Carbon Dioxide and the Reduction of Indophenol and Ferricyanide by Chloroplasts¹

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Summary. Pea chloroplasts isolated in salt media show decreased rates of 2:6 dichlorophenolindophenol (DCPIP) and ferricyanide reduction when depleted of CO₂ at pH values below 7.5. The greatest effect of CO₂ was on uncoupled systems. The incorporation of 10⁻², 2 × 10⁻² and 4 × 10⁻² M sodium acetate into the reaction mixtures progressively increased the bicarbonate concentration required for half maximal rates of reduction of DCPIP. The reaction was saturated by bicarbonate concentrations of 1 to 4 × 10⁻² M. With both DCPIP and ferricyanide, the addition of bicarbonate to illuminated chloroplast systems depleted of CO₂ gave very rapid increases in the rates of reduction. Bicarbonate also stimulated oxygen uptake by the illuminated chloroplasts when added hydrogen acceptors had been reduced. There was no effect of bicarbonate on ferricyanide reduction at low light intensities, but with DCPIP reduction, the apparent magnitude of the effect was independent of light intensity. This suggests that DCPIP reacts with the chloroplast electron transport chain at a site nearer to a photochemical stage than does ferricyanide. It also suggests that CO₂ has at least 2 sites of action.

Illuminated chloroplasts will reduce NADP and other suitable hydrogen acceptors with concomitant ATP production and oxygen evolution. The photo-reduction of hydrogen acceptors with the evolution of oxygen is considered a necessary preliminary to carbon dioxide reduction, but a quite separate process. Thus the current view of photosynthesis is that the fixation of CO₂ is a non-photochemical process which requires reduced NADP and ATP.

An observation by Boyle (4) suggested that CO₂ is necessary for maximum activity in oxygen production by chloroplasts. Warburg and Krippahl showed a requirement for CO₂ in oxygen production resulting from the reduction of quinone and ferricyanide by kohlrahi (*Brassica caulorapa*, Pasq.) chloroplasts. They postulated a new photosynthetic scheme involving a unique mechanism for the action of CO₂ in the photochemical processes involved in oxygen production (23, 24).

Since that time a number of groups of workers have found an effect of CO₂ on measured electron transport using a variety of tissues, electron acceptors and experimental conditions (1, 3, 6, 8, 12, 16, 17, 18, 20, 21). The magnitude of the effect and the conditions producing it show considerable variation.

Punnett and Iyer (16) also showed that bicarbonate can stimulate the rate of phosphorylation more than it stimulates the rates of concomitant ferricyanide or quinone reduction. They used oat chloroplasts not previously deprived of CO₂. Punnett (15) also showed that bicarbonate stimulated FMN- or pyocyanine-catalyzed phosphorylation. Batra and Jagendorf (3) using spinach, showed in addition that bicarbonate decreased the steady state level of X-E, a non-phosphorylated high energy product of the phosphorylating mechanism.

Good (6), Izawa (12), and Batra and Jagendorf (3) using ferricyanide, quinone and pyocyanine-catalyzed phosphorylation respectively, showed a greater effect of CO₂ at high than at low light intensities. They concluded that the CO₂ effect is limited to a non-photochemical process.

The present investigation is concerned with the effect of CO₂ or bicarbonate on the reduction of 2:6 dichlorophenolindophenol (DCPIP). This had not previously been studied although DCPIP has been frequently used as an electron acceptor in illuminated chloroplast systems. We compared this with the effect CO₂ on ferricyanide reduction which was already well documented (e.g. 3, 6, 16, 18, 23).

Most experiments were carried out in the presence of sodium acetate, shown by Good (6) to enhance the effect of CO₂.

It is not possible to use the present results to distinguish between the effects of dissolved carbon dioxide and the bicarbonate ion.

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Materials and Methods

For most experiments, chloroplasts were prepared from leaves of 2 and one-half to 3 and one-half week old pea plants (*Pisum sativum* L. var. Laxton's superb) grown in vermiculite in a greenhouse in ordinary daylight. However, during the winter months the chloroplasts lost activity very rapidly during the relatively long periods of the experiments. A growth chamber following the model of Walker (22) was made. The peas were grown in vermiculite in low light intensities provided by five 40w Ecko Daylight fluorescent tubes on for 12 hours out of every 24. The temperature was $14^{\circ} \pm 1^{\circ}$ during the dark periods and $18^{\circ} \pm 1^{\circ}$ during the light periods. The maximum rates of dye reduction by these chloroplasts were rather lower, but the chloroplasts retained most of their activity during the experiments. No qualitative differences in the responses of the 2 types of chloroplasts to CO_2 /bicarbonate were seen.

Leaves were ground in a chilled mortar in buffer containing 0.35 M NaCl, 0.001 M MgCl_2 , 0.067 M tris-HCl (pH 7.4) (10). The homogenate was filtered through 4 layers of muslin into cold centrifuge tubes and spun at 1100 g for 7 minutes. The pellet was suspended in about one third of the previous volume of buffer and spun at 1100 g for 4 minutes. The pellet was finally suspended in 2 to 5 ml of a mixture of 0.26 M NaCl, 0.01 M sodium acetate, or for some experiments, in 0.26 M NaCl alone. The chlorophyll content was 1 to 2 mg/ml. Chlorophyll was estimated by extracting aliquots in 80% (v/v) acetone and measuring the O.D. at $652 \text{ m}\mu$ (2).

CO_2 was removed from the chloroplast suspension by transferring it to a 250 ml conical flask attached by ground glass joints to a 200 ml round flask containing about 10 ml saturated KOH impregnating a paper fan. The suspension were kept dark and shaken gently in the cold room (4°) for up to 3 and one-half hours. Although such long periods of CO_2 depletion were not normally required when the chloroplasts were treated with acetate/chloride, they were usually given to minimise differences caused by changes in procedure.

From the reaction mixtures, CO_2 was removed by boiling out, or by making up the reagents in CO_2 -free water. Diffusion of CO_2 into the reagents was prevented by soda-lime tubes.

Reactions were aerobic and carried out at $22^{\circ} \pm 1^{\circ}$. Most experiments were done using a final volume of 3 or 5 ml in a medium containing 0.26 M NaCl and 0.01 M Na acetate. For some purposes acetate was omitted or higher concentrations used. The pH was adjusted by 0.1 or 0.16 M phosphate buffers made by mixing Na_2HPO_4 and KH_2PO_4 . Most reactions were run at a measured initial pH of 6.65. The high salt concentrations depressed the pH of the reaction mixtures to the extent of 0.3 to

0.4 pH units below the theoretical value for the phosphate combination used. For most experiments samples of the chloroplast stock suspension were added directly to the reaction mixture. Such reactions are termed to be with whole chloroplasts. Broken chloroplasts were made by adding samples of the chloroplast stock suspension to 2 ml of water. After 3 minutes the reaction mixtures were made up to the same final salt concentrations.

The CO_2 /bicarbonate content of the reaction mixtures was adjusted by the addition of KHCO_3 usually in aqueous solution. For most experiments 4×10^{-2} M bicarbonate was used.

Illumination of the reaction mixtures was by a 500w Aldis slide projector (tungsten lamp) or a 150w Aldis QI 24 projector (tungsten-iodide lamp approximately 20,000 lux) through a 5 cm thick water-filled glass trough.

Light intensities were varied by Balzers neutral density interference filters of fixed percentage transmission. For ferricyanide reduction, a red cut-off filter transmitting light above $590 \text{ m}\mu$ to a maximum of 69% was used in addition. The QI 24 projector lamp gave saturating light even for the DCPIP reductions.

Reduction of DCPIP was measured in an E.E.L. colorimeter using Ilford filter OR1. Five ml samples in 5 ml colorimeter tubes with polythene caps were normally used. Two reaction mixtures, with and without added CO_2 were illuminated simultaneously for each experiment. Readings were taken at 1 minute intervals but staggered by 15 seconds.

Reduction of ferricyanide was measured in a Unicam SP 500 spectrophotometer, by the decrease in optical density of the reaction mixture at $420 \text{ m}\mu$ on illuminating single 1 cm cuvettes through their optical surfaces for successive 1 minute intervals.

In all cases, initial rates of reduction in $\mu\text{moles/mg chlorophyll}\cdot\text{hr}$ are calculated on the basis of the amount of acceptor reduced in the first minute of illumination, although readings were continued for at least 3 minutes often much longer, and progress curves were always plotted.

Oxygen production was measured using an oxygen electrode made by Rank Bros., Bottisham, Cambridge. A polarising voltage of 0.6v was applied to the electrode. The current was recorded automatically by a Honeywell-Brown recorder. The slope of the recorded line gave the rate of production or uptake of oxygen. The 5 ml perspex reaction vessel was illuminated through the transparent cooling jacket. The reaction mixture was rapidly stirred magnetically. Reagents, e.g. KHCO_3 , NH_4Cl could be added from a syringe through a small hole in the lid during the reaction.

To minimize any effect of aging, the relatively longer experiments were arranged so that the conditions were varied in sequence, and the series repeated in the reverse order.

Results

Activity of the Chloroplasts. In saturating light, in the presence of 0.26 M NaCl and 0.01 M sodium acetate, initial rates of DCPIP reduction were 140 to 300 μ moles/mg chlorophyll·hr with CO₂, (4×10^{-2} M KHCO₃) and 80 to 170 without CO₂. For ferricyanide reduction uncoupled from phosphorylation, rates were from 500 to 600 μ moles/mg chlorophyll·hr with CO₂ and 350 to 550 without CO₂. ($O_2 = 2$ DCPIP = 4 FeCN). The magnitude of the CO₂ effect varied from one chloroplast preparation to another.

The rate at which chloroplasts lost activity for DCPIP reduction varied. Some preparations retained most of their activity for a further 3 to 4 hours. In others, activity declined at varying rates but reactions with and without CO₂ were usually similarly affected.

Table I. *Effect of CO₂ on the Rate of DCPIP Reduction by Whole and Broken Chloroplasts*

Reactions in 0.26 M NaCl, 0.01 M Na acetate, 0.16 M phosphate buffer pH 6.65, 0.88×10^{-4} M DCPIP with or without 4×10^{-2} M KHCO₃. Chlorophyll 77 μ g in 5 ml. Saturating light. See Materials and Methods for method of breaking chloroplasts.

No	Initial rate of DCPIP reduction			
	Whole		Broken	
	μ moles/mg chlorophyll·hr			
	+CO ₂	-CO ₂	+CO ₂	-CO ₂
1	248	130	209	89
2	235	96	216	74
3	235	115	192	58
4	254	82	158	53
5	231	70	208	81
Avg	243	119	197	71
Rate + CO ₂	2.04		2.78	
Rate - CO ₂				

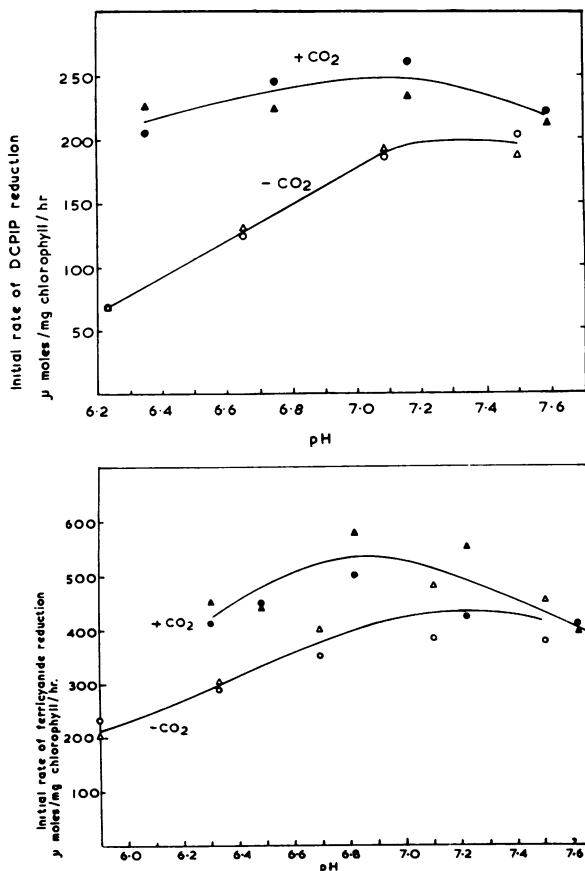


FIG. 1 (top). FIG. 2 (bottom). Effect of CO₂ on DCPIP and ferricyanide reduction at different pH values. Reactions in 0.26 M NaCl, 0.01 M Na acetate, 0.16 M phosphate buffer, with or without 4×10^{-2} M KHCO₃. Saturating light. FIG. 1. 0.88×10^{-4} M DCPIP and 70 μ g chlorophyll/5 ml. FIG. 2. 4.67×10^{-4} M ferricyanide uncoupled with 5×10^{-3} M NH₄Cl, and 42 μ g chlorophyll/3 ml. Duplicate samples shown: 1st values, circles; 2nd values, triangles.

With ferricyanide, activity usually increased slightly during the 3 to 4 hour period necessary to complete the experiments. This may be because the reaction with ferricyanide is more rapid with broken than with whole chloroplasts (see Materials and Methods) in contrast to the rate of DCPIP reduction (cf ref 22 and table I). Although the high salt media may not allow rapid osmotic disruption of the chloroplast membranes, permeability changes would be expected over the long periods of the experiments.

Table I shows the effect of CO₂ on DCPIP reduction by whole and broken chloroplasts. The 2 types of chloroplasts were used alternately throughout the experiment. Rates with broken chloroplasts are lower, but breaking depressed the rate without CO₂ more than the rate with CO₂.

Effect of Changes in pH. Results are shown in figures 1 and 2. Reaction mixtures contained 0.26 M NaCl, 0.01 M sodium acetate, 0.16 M phosphate, with or without 4×10^{-2} M KHCO₃. The added bicarbonate produced a consistent small pH increase, insufficient to account for the CO₂ effect. There was little CO₂ effect above pH 7.5 with either DCPIP, or ferricyanide uncoupled from phosphorylation by 5×10^{-3} M NH₄Cl. The maximum effect with ferricyanide was between pH 6.3 and 7.1. With DCPIP, the effect increased with decreasing pH. However, no correction has been made for the progressive conversion of the dye from the salt to the acid form, so measurements at lower pH values are not reliable. Most other experiments were done at pH 6.65.

Effect of Uncoupling by Ammonium Salts. With ferricyanide, the maximum effect of CO₂ was on uncoupled systems. This is similar to results of Good (6) and Stern and Vennesland (18).

Figure 3 shows recorder traces from the oxygen electrode during a series of experiments on oxygen evolution accompanying ferricyanide reduction. To

alternate samples, NH_4Cl (0.1 ml of 0.2 M) was added before bicarbonate (0.1 ml 2 M), then the procedure reversed. Reagents were added from a syringe during the reduction. Responses were rapid. NH_4Cl had no effect without CO_2 but gave a slight stimulation with CO_2 . Bicarbonate stimu-

lated the rate without NH_4Cl , but had a greater effect if NH_4Cl was already present. Final rates with both together were not significantly different. NH_4Cl stimulated the reduction of ferricyanide by freshly prepared chloroplasts to a much greater extent as they were not already partially uncoupled

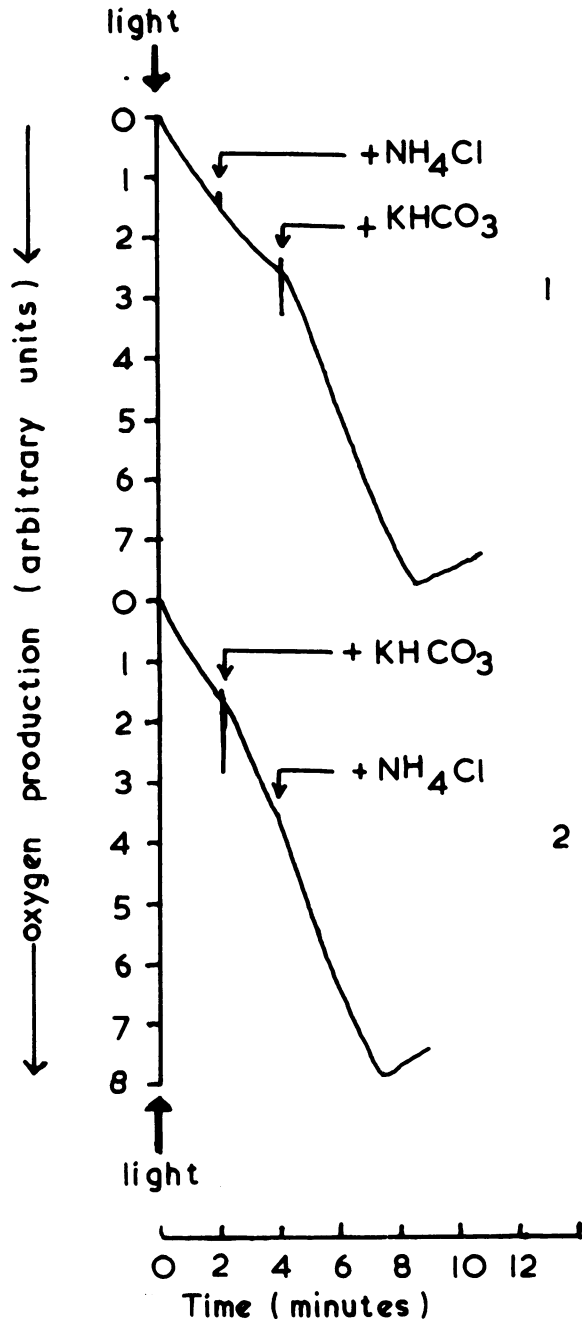


FIG. 3. Effect of KHCO_3 and NH_4Cl on ferricyanide reduction: recorder traces of oxygen production. Reactions in 0.26 M NaCl, 0.01 M acetate, 0.1 M phosphate buffer pH 6.65, 4×10^{-4} M ferricyanide and 90 μg chlorophyll/5 ml. 5×10^{-3} M NH_4Cl added before or after 4×10^{-2} M KHCO_3 . Saturating light.

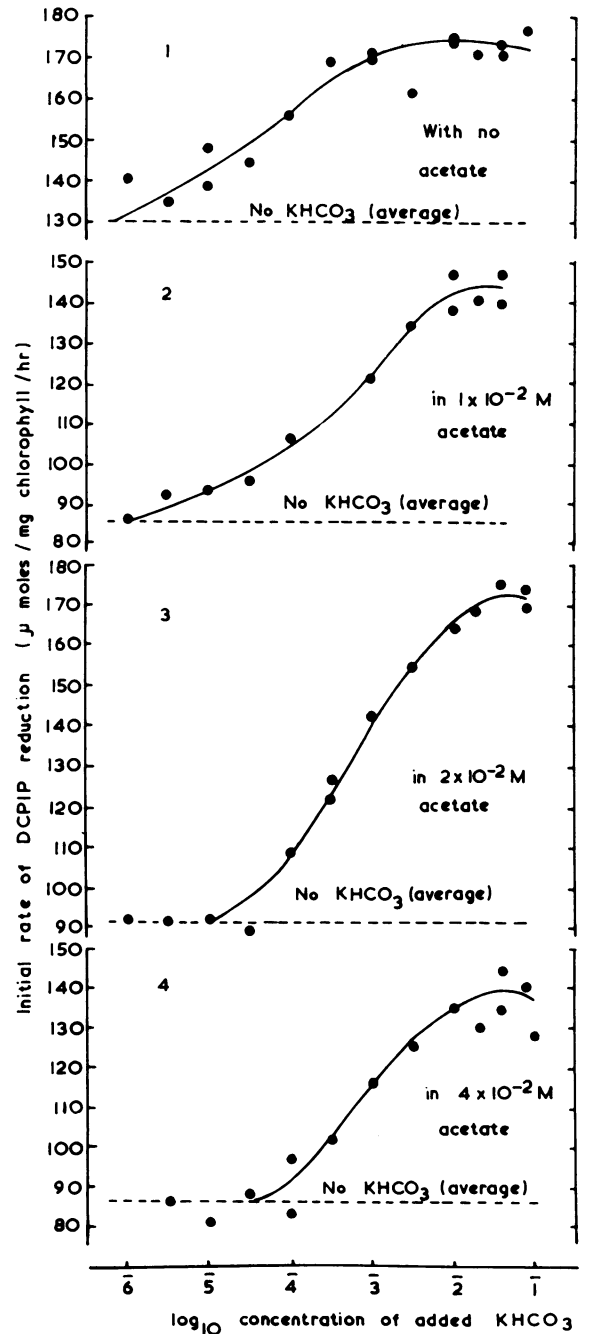


FIG. 4. Effect of KHCO_3 concentration on DCPIP reduction in the presence of 0, 10^{-2} , 2×10^{-2} and 4×10^{-2} M Na acetate. Reactions in 0.26 M NaCl, 0.1 M phosphate buffer pH 6.65, 0.72×10^{-4} M DCPIP. Chlorophyll content/5 ml: no. 1, 81 μg ; 2, 99 μg ; 3, 72 μg ; 4, 87 μg . Saturating light.

by the high concentration of salts (5).

For most other experiments with ferricyanide, 5×10^{-3} M NH₄Cl was used in the reaction mixtures.

With DCPIP, NH₄Cl had no effect on the rate of reduction without CO₂, and little on the initial rate with CO₂, although it shortened the time required for complete reduction. It may be that as the dye is progressively reduced, the uncoupling effect of the oxidized form of the dye itself (7, 13) decreases, so the effect of the ammonium salts increases.

Effects of Acetate. Good (6) made an extensive survey of the reaction conditions influencing the CO₂ effect on oxygen production. He found that the smaller mono-functional ions e.g. formate, acetate, chloride and fluoride increase the dependence on CO₂.

His combination of 0.26 M NaCl and 0.01 M Na acetate in the reaction mixture was used in most of our experiments. It was found however that in this medium, long periods for the removal of CO₂ from the chloroplasts were not always required. When the chloroplast pellet had been in contact with the acetate/chloride mixture for 15 minutes to 1 hour, there was no difference between chloroplasts shaken with KOH and those without. Higher concentrations of acetate gave more pronounced effects in shorter times. Table II shows the rates of reduction of DCPIP when the chloroplasts had been incubated in the complete reaction mixture containing higher concentrations of acetate, for short periods. As was found by Good, (6) acetate depressed the rate of reduction in the absence of CO₂ but had little effect in its presence.

Acetate increased the rate of O₂ uptake by the chloroplast preparations (see section on O₂ uptake).

Acetate also increased the bicarbonate required for half maximal rates of reduction of DCPIP (see next section).

Effect of Bicarbonate Concentration. Figure 4 shows the results of 4 experiments on the effect of different bicarbonate concentrations on DCPIP reduction in the presence of 0, 10⁻², 2 × 10⁻² and

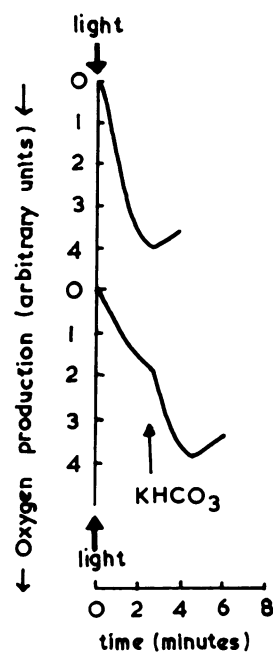


FIG. 5. Recorder traces of oxygen production accompanying DCPIP reduction: effect of adding KHCO₃ initially and during illumination. Reactions in 0.26 M Na acetate 0.1 M phosphate buffer pH 6.65, 0.93×10^{-4} M DCPIP and 87 μg chlorophyll/5 ml.

4×10^{-2} M sodium acetate respectively. In all cases, the chloroplast pellet was suspended in 0.26 M NaCl only. Reaction mixtures were made up in pairs at the appropriate acetate concentration. Chloroplasts were added to each and the contents rapidly mixed. Bicarbonate solution was added to 1 tube and the tubes left for exactly 5 minutes between mixing the chloroplasts and illumination. In this set of experiments results are given after correcting for aging of the material. As the experiments were done on different days and the magnitude of the CO₂ effect varies from 1 chloroplast sample to another, it is not possible to compare the absolute rates found in one experiment

Table II. Influence of Different Acetate Concentrations and Incubation Times on the Effect of CO₂ on DCPIP Reduction

After isolation, half the chloroplasts were suspended in 0.26 M NaCl, 0.01 M Na acetate, the rest in 0.26 M NaCl alone. Both had 3 hrs for CO₂ depletion. Reactions in 0.26 M NaCl, 0.16 M phosphate buffer (pH 6.65), 0.97×10^{-4} M DCPIP, acetate as shown, with or without 4×10^{-2} M KHCO₃. Chlorophyll 62 μg/5 ml. Saturating light.

Time in 0.26 M NaCl	Time in 0.26 M NaCl		Initial rate of DCPIP redn		Ratio +CO ₂ —CO ₂
	+ Na acetate	Na acetate conc	+CO ₂	—CO ₂	
hr		M			
3½	2-3 min	2×10^{-2}	218	146	1.5
4	"	4×10^{-2}	232	97	2.4
4½	10 min	2×10^{-2}	177	43	4.1
4¾	"	4×10^{-2}	145	33	4.4
0	5½ hr	1×10^{-2}	211	105	2.0
6	0	0	192	142	1.35

with those of another. In all cases, the system was sensitive to a very wide range of bicarbonate concentrations. The apparent requirement for bicarbonate increased with increasing acetate concentration. In the absence of acetate, half maximal rates were given by about 2×10^{-4} M KHCO_3 . This increased to about 10^{-3} M with 4×10^{-2} M acetate. The systems were saturated by 1 to 4×10^{-2} M KHCO_3 . This is a higher requirement than that found by most workers.

Stern and Vennessland (17) and Good (6) found that CO_2 removal is reversible if CO_2 is added before illumination. Stern and Vennessland (18) found an average of 79% restoration of activity towards ferricyanide reduction. Using DCPIP we found that maximal rates were usually completely restored if bicarbonate, either solid (9) or in solution, was mixed or injected into the CO_2 -depleted reaction mixture during illumination. This can be seen in figure 5. Rates stimulated by bicarbonate above those of controls where bicarbonate was added initially, were frequently seen.

The response of CO_2 -free systems to added bicarbonate was very rapid (figs 3 and 5).

Effect of Light Intensity. Figures 6 and 7 show the rates of DCPIP reduction and ferricyanide reduction uncoupled from phosphorylation, with and without CO_2 , at different light intensities. With ferricyanide, there is no CO_2 effect at very low light intensities. With DCPIP, absence of CO_2 limits the rate whatever the light intensity and the apparent magnitude of the effect is independent of light intensity.

Oxygen Uptake. Experiments using the oxygen electrode indicate that there is a considerable oxygen uptake by the illuminated chloroplast reaction mixtures as soon as the ferricyanide or DCPIP is completely reduced (figs 3, 5, 8). Rates of oxygen

uptake are more rapid in the presence of bicarbonate.

Figure 8, of recorder traces from the oxygen electrode shows the effects of 4×10^{-2} M KHCO_3 and 1×10^{-2} M sodium acetate on oxygen production and uptake using DCPIP. Bicarbonate increases the rate of oxygen uptake whether added before or after the reduction. There is less difference in acetate-free systems.

Discussion

The existence of a requirement for CO_2 (or bicarbonate) for maximal rates of O_2 production and reduction of hydrogen acceptors by illuminated chloroplast systems is well established. The extent of the effect and the conditions producing it have been shown to be very variable.

Quinone and ferricyanide as hydrogen acceptors have been frequently used (e.g. 1, 3, 6, 12, 18, 24) also FMN (6) and the natural hydrogen acceptor NADP (18).

Punnett and Iyer (16) as distinct from many other investigators studied phosphorylating systems with chloroplasts not depleted of CO_2 . They found a stimulatory effect of bicarbonate on phosphorylation accompanying quinone and ferricyanide reduction with an increase in the $P/2_e$ ratio. Punnett (15) subsequently found an effect of bicarbonate on phosphorylation with FMN and pyocyanine. The greatest effect of bicarbonate was on pyocyanine-catalyzed phosphorylation.

Stern and Vennessland (18) and Good (6) found that the greatest effect of CO_2 on O_2 production was on systems uncoupled from phosphorylation. Batra and Jagendorf (3) found that bicarbonate decreases the steady state level of X-E, which term they used to describe the high energy state of

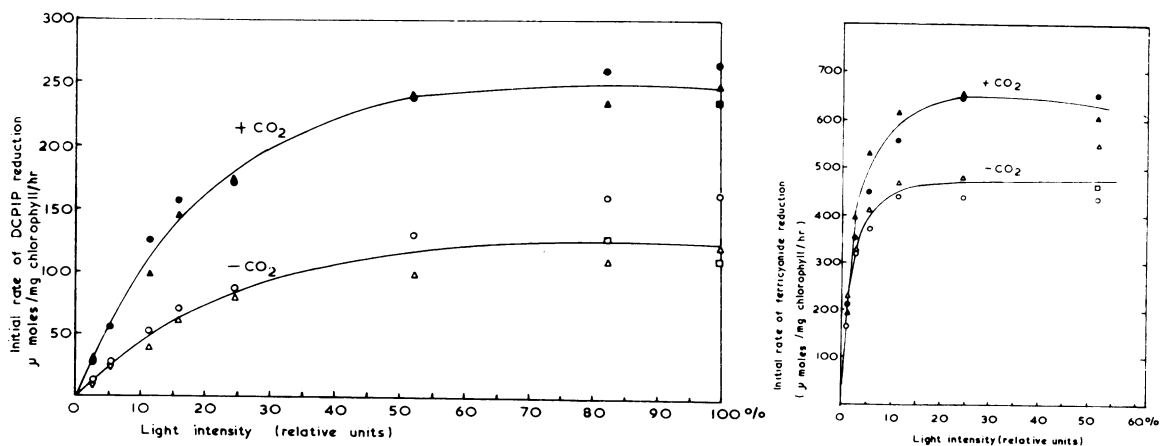


FIG. 6 (left). FIG. 7 (right). Influence of light intensity on the effect of CO_2 on DCPIP and ferricyanide reduction. Reactions in 0.26 M NaCl, 0.01 M Na acetate, 0.1 M phosphate buffer pH 6.65. FIG. 6. 0.88×10^{-4} DCPIP and 62 μg chlorophyll/5 ml. FIG. 7. 4.67×10^{-3} M ferricyanide with 5×10^{-3} M NH_4Cl and 38 μg chlorophyll/3 ml. Illumination by Q1 24 projector with Balzers neutral density filters. Duplicate samples shown: 1st values, circles; 2nd values, triangles; 3rd values, squares.

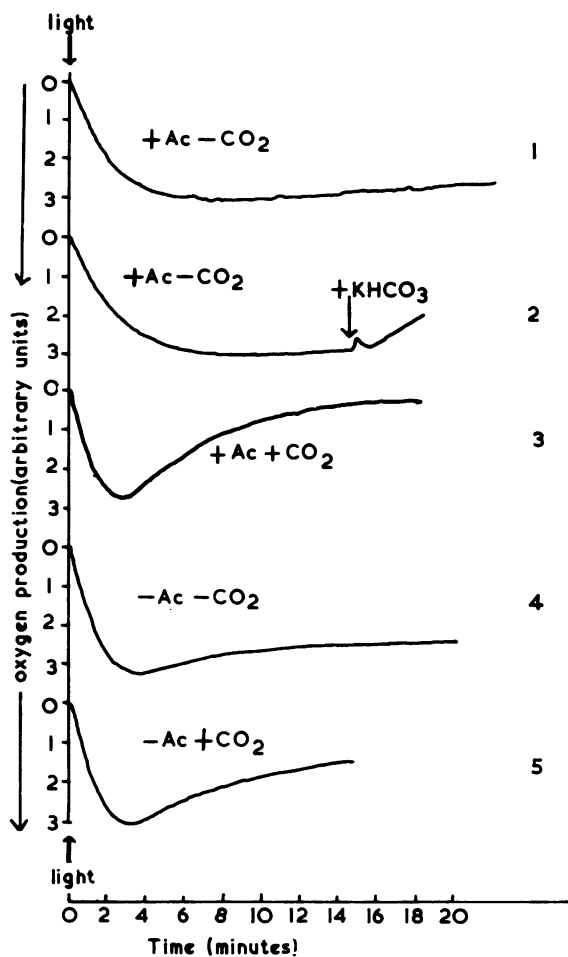


FIG. 8. Effects of acetate and KHCO_3 on oxygen production and consumption: recorder traces from oxygen electrode using DCPIP. Reactions in 0.26 M NaCl , $0.1 \text{ M phosphate buffer pH } 6.65$, $0.88 \times 10^{-4} \text{ M DCPIP}$ with or without $10^{-2} \text{ M Na acetate}$ and $4 \times 10^{-2} \text{ M KHCO}_3$. Chlorophyll content $80 \mu\text{g}/5 \text{ ml}$.

chloroplasts immediately following illumination in the presence of a hydrogen acceptor (11).

Batra and Jagendorf compared the methods of Punnett and Iyer with those of Good (6). They concluded that there are 2 effects of CO_2 (or bicarbonate). The CO_2 requirement originally discovered by Warburg (23,24) affects the electron transport processes involved in the production of O_2 . The CO_2 effect found by Punnett and Iyer can operate in the absence of O_2 production and may be interpreted as being concerned with part of the phosphorylating mechanism. Their evidence can be summarized: 1) The stimulatory effect of bicarbonate on measured electron transport found by Punnett and Iyer is not shown in the presence of optimal concentrations of uncoupling agents; e.g. ethylamine. 2) The range of added bicarbonate required is different in the 2 systems. The

range of the effect on oxygen production was between 0.2 and 2 mM, while that found by Punnett and Iyer was between 1 and 8 mM. 3) The effect observed by Punnett also applied to pyocyanine-catalyzed phosphorylation. This system is not inhibited by CO_2 removal but is stimulated by added bicarbonate even when the O_2 evolving mechanism is inhibited by 10 mM CMU (*p*-chlorophenyl-1,1-dimethyl urea).

The present work refers to the effect of differing concentrations of CO_2 on photo-induced electron transport processes which depend on O_2 evolution. In common with Good (6) and Stern and Vennessland (18) we find the greatest effect of the addition of bicarbonate on ferricyanide reduction when the system is uncoupled from phosphorylation.

With ferricyanide at low light intensity, the rate of reduction is independent of removal and addition of bicarbonate. This is what was found by Good. The same situation appeared to hold in the reduction of quinone, which was found by Izawa to show a much greater effect of CO_2 at the higher light intensities. Both Good and Izawa concluded therefore that the CO_2 effect is confined to a rate-limiting thermochemical process. In contrast to this, we find that, using DCPIP, lack of CO_2 limits the rate at the lowest intensities at which the reduction of the dye can be measured.

The simplest explanation would seem to be that under the conditions of our experiments, DCPIP accepts hydrogen equivalents at a site nearer to a primary photochemical step than does ferricyanide.

If we accept the current scheme of 2 light reactions coupled by a dark reaction involving an oxido-reduction sequence, then it would seem that both reagents appear to depend only on reaction II for their reduction. The DCPIP ($E'_0 + 0.2 \text{ v}$) might be mainly reduced near to plastoquinone (E'_0 about zero v) and the ferricyanide ($E'_0 + 0.4 \text{ v}$) reduced nearer to cytochrome *f* (E'_0 about $+ 0.37 \text{ v}$). (These characteristic oxidoreduction potentials refer to pH 7.0.)

The bicarbonate concentrations ($1-4 \times 10^{-2} \text{ M}$) required to saturate our system even in the absence of acetate are greatly in excess of those found by other workers (3,6,18,24) and summarized by Punnett and Iyer. We cannot explain these differences.

Oxygen uptake by chloroplasts has not been considered to be a very general phenomenon. If it occurs it is usually attributed to a Mehler reaction (14). Abeles Brown and Mayne (1) used a mass spectrometer to investigate the effect of CO_2 on quinone reduction by kohlrabi chloroplasts. They found a simultaneous production and consumption of oxygen when the quinone had been reduced. This was taken as evidence of a Mehler reaction. They sometimes found a small oxygen uptake during quinone reduction in the presence or absence of CO_2 . However, these values were

within the experimental error of the method and they concluded that the CO₂ effect was specifically on oxygen production. The problem of the high rates of oxygen uptake by our chloroplast preparations which is stimulated by acetate and bicarbonate is under investigation.

It may be that CO₂ or bicarbonate affects the efficiency with which external hydrogen acceptors and donors can react with the endogenous electron transport chain. The responses of different chloroplast preparations to the presence and relative absence of CO₂ have been found to show variations both in character and magnitude. In fact, Heise and Gaffron (8) already concluded that metabolic disturbances caused by a deficiency of CO₂ are a very general phenomenon and not restricted to photosynthesis. We do not consider that there is enough information available at present, to allow a detailed interpretation of the effects observed with the various experimental systems. In the present work, the difference in the effects of light intensity on the reduction of ferricyanide and on the reduction of DCPIP would suggest that the CO₂ is affecting at least 2 different locations in the photochemically active particles from the plant.

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