

STIMULATION OF THE HILL REACTION BY CARBON DIOXIDE^{1, 2}

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Hill reactions (i.e., O₂ evolution by illuminated chloroplast preparations) are generally assumed to represent the photochemical splitting of water. For about 20 years the view has prevailed that, in the course of a Hill reaction, photolysis generates an O₂ precursor and a reductant which is capable of reacting directly or indirectly with any of a variety of oxidants. The metabolism of both photolysis products (oxidant & reductant) evidently is enzymatically controlled. The list of oxidizing agents effective in the Hill reaction began chronologically with ferric oxalate and now includes an impressive number of naturally-occurring as well as exotic substances. On the assumption that oxygen evolution and oxidant reduction are separate if not independent processes, there is no compelling a priori reason to believe CO₂ is uniquely involved in the Hill reaction even in a catalytic capacity. However, because of the close analogy between photosynthesis and the various types of Hill reactions, the possible role of carbon dioxide in chloroplast reactions continues to be a subject of concern both theoretically and experimentally, especially so because several reports in the literature suggest that CO₂ at least in some instances may have a catalytic effect on reaction sequences which involve water photolysis.

Franck (7) found an influence of CO₂ on the evolution of O₂ by chloroplasts prepared from tobacco leaf. He studied a Molisch-type reaction in which no Hill oxidant was added to the preparations. Only small amounts of O₂ were evolved but the rate was significantly higher when CO₂ was present. Brown and Franck (3) using C¹⁴O₂ found that carbon 14 was not taken up under the conditions of these experiments. They concluded that CO₂ could play only a catalytic role.

Kessler (9, 10) observed that the rate of reduction of nitrite by illuminated *Ankistrodesmus* cells as measured by H₂ uptake was increased in the presence of catalytic amounts of CO₂. Experiments performed with C¹⁴O₂ showed that the reduction of nitrite with hydrogen was not accompanied by an appreciable

fixation of carbon. Thus the effect of CO₂ apparently was indirect.

Boyle (2) reported manometric experiments from which he claimed at least small quantities of carbon dioxide were required for O₂ evolution in the light using triturated spinach leaves. These experiments were repeated by Clendenning and Gorham (6) who found no differences in Hill reaction rate between vessels which contained carbon dioxide and those which did not. Because Boyle's results were not confirmed and besides were incompatible with prevailing theory they have generally been ignored.

Warburg and Krippahl (11, 12) using kohlrabi chloroplast preparations with quinone as the oxidant reported that small amounts of CO₂ increased the rate of the Hill reaction. Similar effects were noted with frozen-dried *Chlorella* and with quinone-poisoned whole *Chlorella* cells. Such results are of course reminiscent of the earlier observations by Boyle, even though the experimental material and methods were different.

It is patently desirable to verify the claim that CO₂ can augment the Hill reaction using experimental methods which adhere closely to those of Boyle and of Warburg and Krippahl. We report here the results of such attempts at confirmation. It was found possible to obtain manometric measurements which were in all respects identical with those reported by Boyle although, as will be shown, these results do not support his interpretation that there was a CO₂ effect on the Hill reaction. Manometric results also were obtained which were at least qualitatively similar to the findings of Warburg and Krippahl with kohlrabi chloroplasts. The reactions in which an apparently valid CO₂ effect could be observed also were measured by mass spectrometry which has the important advantage over manometry of relatively unambiguous analytic specificity. Additional experiments were performed with tracer CO₂ and with tracer O₂ which permitted the detection and measurement of simultaneous production and consumption of these gases thus providing detailed information of the nature of the CO₂ effect. This method could reveal the operation of a cyclic regeneration of CO₂ by illuminated kohlrabi chloroplast preparations.

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MATERIALS & METHODS

Triturated spinach (*Spinacia oleracea* L.) leaves used in repetitions of Boyle's experiment were pre-

pared according to the method described by him. (2)

Chloroplasts and chloroplast fragments were obtained from kohlrabi (*Brassica caulorapa* Pasq.) leaves according to the method described by Warburg (11) which consists of grinding the leaves in a waring blender and using differential centrifugation to obtain chloroplast preparations. Microscopic examination of the preparation obtained by this method showed the presence of both whole and fragmented chloroplasts. In both manometric and mass spectrometer experiments the chloroplast preparations were suspended in 0.01 M KCl.

For certain mass spectrometer experiments frozen chloroplast preparations were used. These were thawed immediately before each experiment. Freezing chloroplast preparations suspended in water destroyed their subsequent activity. However suspending chloroplast preparations in 0.5 M sucrose before freezing gave preparations which upon thawing, centrifugation, and resuspension in 0.01 M KCl had the same activity as non-frozen preparations. Such preparations also exhibited a comparable CO₂ effect on the Hill reaction rate.

Quinone was purified by sublimation before each experiment.

Chlorophyll concentration was determined spectrophotometrically by the method of Arnon (1).

Manometric experiments were performed in the conventional manner. Vessels were shaken at 120 oscillations per minute at an excursion of 4.7 cm in a water bath at 20° C.

In manometric experiments light was supplied by a bank of six 300 photoflood bulbs 22 cm from the bottom of the Warburg vessels. These bulbs were run on 90 volts. In mass spectrometer experiments the light source was a 1,000 w condensed filament Mazda flood light with the image of the filament projected onto the plane of the vessel. The applied voltages are indicated in the data presented.

Experiments with a mass spectrometer were performed using a Nier-Consolidated instrument (Model 21-201) which had been adapted for continuous monitoring of the gas phase of the reaction vessel. This was a rectangular Warburg-type vessel of 15.8 cc total volume when attached to the joint which was in turn connected to the mass spectrometer inlet leak.

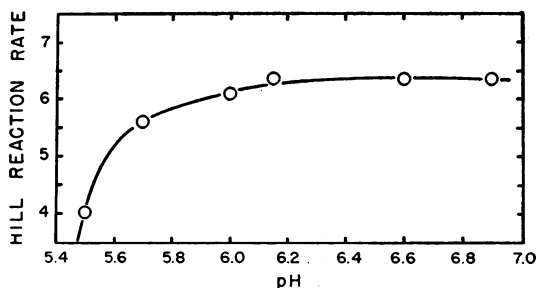
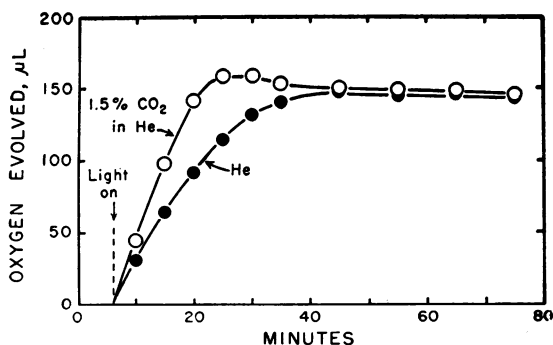
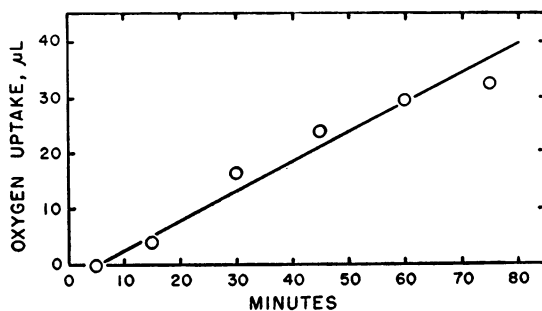
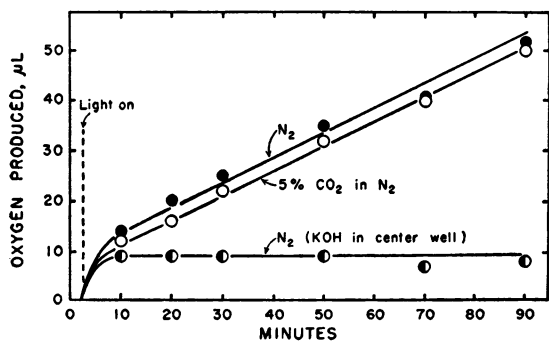


FIG. 1 (upper left). Time course of pressure changes in manometer vessels with and without CO₂ and with KOH in center well. Spinach chloroplast preparation and experimental conditions as specified by Boyle (2).

FIG. 2 (upper right). Time course of oxygen uptake in aerobic control vessel. Quinone in solution in main compartment; KOH in center well; chloroplast material omitted.

FIG. 3 (lower left). Oxygen production by Hill reaction. Kohlrabi chloroplast preparation, 2.5 mg chlorophyll per vessel. Quinone, 2.0 mg. Suspending medium, 0.01 M KCl. Liquid volume, 2.2 ml. Gas phase, helium ± 1.5% CO₂.

FIG. 4 (lower right). Influence of pH on rate of Hill reaction. Kohlrabi chloroplasts suspended in phosphate buffer, 0.05 M.

The design of the joint and leak assembly and the method of shaking the vessel in a constant temperature bath did not differ in any essential particular from what was illustrated previously (8). The use of the mass spectrometer as adapted for the required measurements has been described (4). In those experiments in which tracers were used, isotopically enriched O₂ or CO₂ was introduced initially into the gas phase of the experimental flask; continuous monitoring of the gas phase with the mass spectrometer was carried on thereafter.

Brown and Weis (5) described the computations required to convert data obtained in the form of mass peak heights on the recorder chart to absolute measurements of gas evolution or uptake rates. This computation procedure includes the determination of isotope ratios of gases in solution from data obtained from gas phase measurements and takes into account the effects of diffusion lag of O₂ across the gas-liquid interface.

Argon was added to the gas phase in all mass spectrometer experiments and was monitored continually to facilitate correcting for machine drift and any other pressure changes due to non-biological factors.

RESULTS

Using the triturated spinach leaf preparations described by Boyle, it was not possible to obtain an effect on the rate of the Hill reaction attributable to the presence or absence of CO₂. The rate was the same in 5% CO₂ in N₂ as in 100% N₂ (fig 1). When KOH was added to the manometer vessel center wells however, no pressure change occurred. This fact was found not to be due to the absence of

CO₂ but rather to the reabsorption of all the O₂ evolved by the Hill reaction in the center wells. It is known that quinone will distill from the main compartment of a reaction vessel into the KOH solution. When this was measured quantitatively it was found that in an hour about 34 μg of quinone (1.7% of total) was lost from the main compartment. Furthermore it was observed that, when quinone was absorbed on KOH-soaked filter paper in the center well under aerobic conditions, O₂ uptake occurred. The rate of uptake was found to be 29 μl O₂ per hour under conditions which simulated Boyle's (see fig 2). This rate was approximately equivalent to the rate of the Hill reaction as reported by Boyle and confirmed in the present study. Boyle's method of preparing chloroplast suspensions yielded preparations of low activity. He also used a remarkably high quinone concentration. Accordingly manometer vessels containing quinone in the main compartment, KOH in the center well, and air as the gas phase can be expected to show a rate of O₂ uptake sufficient to reabsorb all the oxygen produced by a Boyle-type Hill reaction. As shown in figure 1, it was possible to reproduce both qualitatively and quantitatively the results reported by Boyle. Nevertheless such results were spurious for the reason indicated.

Figure 3 shows the results of a manometric experiment with a kohlrabi chloroplast preparation designed to duplicate the conditions described by Warburg and Krippahl. These conditions were: 3 ml liquid volume, 0.1% KCl, 2.1 mg quinone, and argon ± 1.4% CO₂. Warburg and Krippahl provided no indication of the amount of chloroplasts added, the pH of the medium, or the use of a buffer. The contents of our vessels are described in the legend of figure 3. The light was turned on as soon as the

TABLE I
CARBON DIOXIDE EFFECT ON RATE OF HILL REACTION DETERMINED BY WARBURG MANOMETRY*

EXPERIMENT NO.	% CO ₂ IN He	CHLORO-PHYLL mg/l	RATE**	%*** DIFF.	% OF THEORETICAL YIELD	%*** DIFF.	FINAL pH OF SUSPENSION
1a	0.001	560	5.75		79.5		6.20
1b	5.0	560	8.35	+45.2	83.5	5.0	5.80
2a	0.001	680	2.57		82.5		6.00
2b	1.5	680	4.71	+83.5	85.0	3.0	6.00
3a	0.001	432	7.05		82.0		6.15
3b	1.5	432	10.7	+51.8	85.5	4.3	6.00
4a	0.001	1116	2.64		74.0		6.05
4b	1.5	1116	4.10	+55.2	79.3	7.2	5.95
4c	0.001	1116	2.18		78.0		6.00
4d	1.5	1116	3.23	+47.9	84.5	8.3	5.90

* Liquid volume, 2.2 ml. Shaking rate, 120 oscillations/min. Excursion of vessel, 4.7 cm. Expected yield on the 2 mg of quinone per flask, 198 μl O₂. Temperature, 20° C. Data are averages for three vessels.

** μl O₂ minute⁻¹ mg chlorophyll⁻¹.

*** Percent differences calculated as:

$$\frac{\text{avg. value for 3 vessels with CO}_2}{\text{avg. value for 3 vessels without CO}_2} \times 100$$

vessels had equilibrated and quinone had been tipped in from the side arm³. In this particular experiment the Hill reaction rate was 55% higher in the presence of CO₂ than in its absence. The total oxygen yield was 7% greater in the presence of CO₂. This latter difference was small but significant. (The average deviation of manometric results was less than 5%.) The data illustrated in figure 3 are representative of a series of tests using different chloroplast preparations. Table I summarizes the results of five such experiments. The data are the averages of three vessels with CO₂ and of three without. The CO₂ content of the helium used to flush the vessels was measured with the mass spectrometer and shown to contain 0.001% CO₂.

Because it had been found that a CO₂ effect on the Hill reaction could not be demonstrated consistently in the presence of phosphate, the experiments of table I were run without added buffer. Since changes in pH could lead to CO₂ retention or evolution thus influencing the pressure change, the pH was measured both before and after each manometric run. Chloroplast suspensions of course are buffered to some extent endogenously. Measured pH changes varied from zero to 0.4 units. At the CO₂ partial pressures which prevailed, such small changes in pH were quite insufficient to account for the differences observed manometrically between vessels which were provided with CO₂ and those which were not.

Furthermore the pH sensitivity of the Hill reaction itself also was not adequate to account for the difference in observed rates due to the presence of CO₂. A pH curve for the Hill reaction by kohlrabi chloroplasts is shown in figure 4. Flushing the vessels with gas containing CO₂ could be expected to cause some small decrease in pH of the suspension. This amounted to about 0.1 pH unit. At a pH of 6.0 to 6.2, according to the relation shown in figure 4, a change of 0.1 unit should have resulted in a barely detectable decrement in the Hill reaction rate rather than in an increase. Thus the CO₂ effect could not be attributed to the small pH changes which occurred in the poorly buffered suspensions.

³ While this manuscript was in preparation a preliminary communication by Stern and Vennesland appeared. (J. Biol. Chem. 235:PC51-53. 1960.) These workers noted that the photochemical reduction of ferricyanide with catalytic amounts of dichlorophenolindophenol by spinach or kohlrabi chloroplasts could be made dependent on the presence of CO₂. The Stern and Vennesland effect seems to be different from the CO₂ effect of Warburg and Krippahl since Stern and Vennesland found a dark incubation time of several hours in the absence of CO₂ was required to elicit the effect. During the long incubation period Hill reaction activity declined with time more rapidly in the absence of CO₂ than in its presence. The addition of CO₂ quickly restored most of the Hill reaction activity. In our studies of kohlrabi chloroplasts using quinone as Hill oxidant rather than ferricyanide, prolonged incubation in the absence of CO₂ did not enhance the CO₂ effect.

The yield of O₂ was slightly but consistently larger when CO₂ was present than when absent (table I). Measurements of yield were taken as the highest point of the oxygen evolution time course.

The stimulatory effect of CO₂ on the Hill reaction also was observed using mass spectrometry in the place of manometry. Two sets of results are presented in table II. Aliquots of the same chloroplast preparation were used in all experiments within a set. Since these observations could not be replicated simultaneously with the apparatus presently available, it was necessary to perform successive experiments on comparable chloroplast samples. This necessitated preservation of Hill reaction activity by holding aliquots of the preparations in the frozen state until just before use. Within each set of experiments shown in table II the stimulatory effect of CO₂ was apparent. Because of differences in activities of chloroplast suspensions prepared at separate times from different sets of leaves as well as differences in

TABLE II
CARBON DIOXIDE EFFECT ON RATE OF HILL REACTION
DETERMINED BY MASS SPECTROMETER*

EXPERIMENT NO.	% CO ₂ IN He	RATE**	% OF THEORETICAL YIELD
<i>Set A</i>			
5a	0.001	6.90	94.0
5b	0.003	6.05	74.0
5c	0.002	9.05	101.
5d	0.003	7.40	97.7
		Avg. 7.35	
5e	0.38	8.65	96.0
5f	0.14	8.65	95.5
5g	0.27	8.60	97.5
5h	0.72	8.95	101.
		Avg. 8.71	
<i>Set B</i>			
6a	0.0005	3.03	85.1
6b	0.001	2.93	84.3
6c	0.001	2.98	81.4
		Avg. 2.75	
6d	0.58	4.25	84.5
6e	0.82	4.47	85.6
6f	0.61	4.32	85.5
		Avg. 4.35	
* Conditions:		<i>Set A</i>	<i>Set B</i>
	Temperature	17° C	15° C
	Chlorophyll conc.	344 mg/l	772 mg/l
	Voltage supplied to light source	80 volts	110 volts
		** $\mu\text{l O}_2 \text{ minute}^{-1} \text{ mg chlorophyll}^{-1}$	

incident light intensity and chloroplast density, the rates of oxygen evolution in sets A and B are different. These data demonstrate convincingly that O₂ was indeed the only gas involved since the analytical method readily distinguished it from CO₂ and other gases.

Mass spectrometer experiments were carried out under aerobic and anaerobic conditions with and without CO₂. In all experiments the level of CO₂ initially present in the vessel remained unchanged. For those experiments in which tracer CO₂ was present the production and consumption rates were calculated independently from the tracer data. In all cases neither evolution nor uptake rates differed significantly from zero (table III).

It is difficult to present a single measure of reliability, but 10% may be considered the limit of error of values computed from mass spectrometer data such as those presented in table III.

After the quinone had been reduced in the Hill reaction a simultaneous production and consumption of oxygen was observed. This was evidence for a Mehler reaction which would be expected under these conditions.

Some of the experiments in table III demonstrate an uptake of oxygen during the course of quinone reduction. Since these results fall within the range of error of the mass spectrometer method, it can be concluded that this does not contribute to the stimulatory effect of carbon dioxide on the rate of the Hill reaction. Besides, an uptake can be noted in both the presence and absence of carbon dioxide.

Warburg and Krippahl interpreted the CO₂ effect

they had observed by reviving the discredited hypothesis that O₂ arises from the splitting of CO₂ by light. According to them the C resulting from that hypothetical cleavage reacts with quinone and water to form hydroquinone and CO₂ which again can be split and recombined in cyclic fashion. To test the hypothesis that the catalytic effect of CO₂ was achieved by a mechanism which involved the cyclic uptake and production of this gas by the chloroplast preparations, experiments were performed using CO₂ labelled with carbon 13. When a mixture of C¹³O₂ and C¹²O₂ was present in the gas phase, no consumption or production of either isotopic form of CO₂ was observed (table III).

Even though no cyclic metabolism of CO₂ could be detected, it must be realized that this in itself does not rule out the possibility that the environmental CO₂ was passing through a metabolic pool of chloroplast fixed carbon. So long as the turnover of such a hypothetical pool involves the production of the same carbon atoms which are incorporated, such a cyclic process could escape detection provided that the pool size is too small for changes to be observed in the isotopic ratio of the labelled CO₂. From the studies carried out with tracer carbon, it was calculated that a hypothetical pool of metabolic carbon compound could amount to no more than ½% of the chlorophyll present and still escape detection in such experiments. On the other hand, if the hypothetical pool should equilibrate by rapid exchange with environmental CO₂ then it follows that an even larger pool would not be directly detectable by tracer experiments such as were performed.

TABLE III
PRODUCTION & CONSUMPTION OF OXYGEN & CARBON DIOXIDE DURING
HILL REACTION DETERMINED BY MASS SPECTROMETRY*

GAS PHASE	EXP. NO.	CHLORO-PHYLL. CONC.	% O ₂ , GAS PHASE	% CO ₂ , GAS PHASE	O ₂ , NET RATE OF CHANGE	O ₂ PRO-DUCTION RATE	O ₂ CON-SUMPTION RATE	CO ₂ NET RATE OF CHANGE	CO ₂ PRO-DUCTION RATE	CO ₂ CON-SUMPTION RATE
Helium	7a	954	1.55	0.0
	7b	954	2.85	0.0
Helium	8a	410	...	0.18	3.25	0.015	0.18**
CO ₂ ***	9a	1280	...	1.11	3.25	0.015	0.0
	9b	1280	...	0.33	3.56	0.0	0.0
Helium	10a	258	0.27	7.25	0.62**	0.0
O ₂ †	10b	128	0.49	5.15	-0.63	0.0
Helium	8b	410	0.35	1.15	...	4.42	-0.05	...	0.04	-0.09
CO ₂ ***	10c	128	0.53	0.73	...	3.48	-0.56	...	0.08**	0.0
O ₂ †										

* Temperature, 15° C. Voltage to light, 80. Rates are expressed as $\mu\text{l O}_2 \text{ min}^{-1} \text{ mg}^{-1}$ chlorophyll. In all cases gas evolution is recorded as +, gas consumption as -.

** Whenever calculations yield positive values for gas uptake or negative values for production, these are examples of experimental error.

*** Mixture of mass 44 and 45.

† Mixture of mass 32 and 34.

CONCLUSIONS

Boyle's observations with triturated spinach leaves were confirmed, but for the reasons discussed, such experiments do not demonstrate a catalytic CO₂ effect on the rate of the Hill reaction.

It was possible to obtain the catalytic effect of CO₂ described by Warburg and Krippahl though our results were quantitatively not as large. This effect is specific for oxygen evolution. There was no demonstrable production or consumption of CO₂ under conditions for which a CO₂ effect was observed. This finding fails to support but does not disprove the hypothesis that the CO₂ effect involves a rapid turnover of carbon.

An augmentation of the Hill reaction could not be noticed in experiments where both 0.5 M sucrose and 0.05 M phosphate buffer were present. Where phosphate buffer was used alone the CO₂ effect was reduced to about half of that of the 0.01 M KCl control. For vessels containing 0.5 M sucrose a CO₂ effect was demonstrable though the results were variable as compared to the control.

Sugar beet (*Beta vulgaris* L.) chloroplasts prepared in the same manner as the kohlrabi chloroplasts did not show a significant stimulation of the Hill reaction by CO₂. This CO₂ effect, therefore, is patently not as general a phenomenon as is Hill reaction activity itself.

SUMMARY

Experiments by Boyle purporting to show a CO₂ stimulation of the Hill reaction were repeated. While Boyle's results were confirmed they were considered not to be evidence of a CO₂ effect per se. When alkali *was not* present in vessel center wells, the same rate of Hill reaction occurred with or without CO₂. When alkali *was* present, some quinone distilled from the main compartment onto the alkali-soaked filter paper in the center wells. The resulting mixture consumed O₂ at a rate comparable with the rate of O₂ production in the Hill reaction.

A report by Warburg and Krippahl of a CO₂ stimulation of the Hill reaction similar to that of Boyle but using different materials and methods was confirmed. With manometry and experimental conditions as specified by Warburg and Krippahl 1.5 to 5% CO₂ was found to enhance the rate of the Hill

reaction by approximately 50%. Using mass spectrometry it was established that the CO₂ effect was specifically on the rate of O₂ evolution, not on O₂ uptake; nor was a gas other than O₂ evolved. No metabolism of CO₂ was observed. Using tracer CO₂ it was not possible to demonstrate a cyclic evolution-uptake of CO₂ which had been postulated by other investigators.

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