Phytochemistry, 1966, Vol. 5, pp. 1013 to 1019. Pergamon Press Ltd. Printed in England

EXPERIMENTS ON THE ORIGIN OF CO_2 RELEASED BY TOBACCO LEAF SEGMENTS IN THE LIGHT

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(*Received* 17 *November* 1965)

Abstract—Tobacco leaf segments were allowed to incorporate $^{14}CO_2$ in the light, and then subjected to an air stream lacking the isotope. The release of $^{14}CO_2$ and total CO_2 were followed during the latter period. The specific activity of the CO_2 released into CO_2-free air was higher in light than in darkness. This suggests that illumination brings about a change of substrate for CO_2 production, recent assimilate being preferentially oxidized in the light. Less $^{14}CO_2$ was released in the light into air containing 300 ppm $^{12}CO_2$ than into CO_2-free air. External $^{12}CO_2$ at this concentration, however, had no effect on the rate of $^{14}CO_2$ release in darkness. The release of CO_2 in the light was greatly stimulated by high oxygen concentration, whereas the rate of CO_2 release in the dark was virtually unaffected. The above results are used as evidence that different mechanisms are involved in the release of CO_2 in light and darkness. The presence of either of two inhibitors of glycollate metabolism, namely α-hydroxy-2-pyridine-methane sulphonate and iso-nicotinyl hydrazide, reduced the specific activity of the CO_2 released in light to a level characteristic of that released in darkness. This, together with the effect of high oxygen, is taken as evidence in support of a hypothesis that much of the CO_2 released by the leaf in light is derived by the oxidation of glycollate.

INTRODUCTION

IT HAS long been established that the green parts of plants take up oxygen and release carbon dioxide in the light, a process which has been termed *light respiration.* It is not easily measured, however, due to the simultaneous occurrence of photosynthesis which carries out the reverse overall reaction. Nevertheless, light respiration has been shown to occur by the use of a number of different techniques.

Oxygen absorption in the light has been followed in the Warburg apparatus under CO_2-free conditions when photosynthetic oxygen production was minimal.[1] In addition, work using the tracer ^{18}O has demonstrated an uptake of oxygen under conditions when photosynthesis was causing an overall oxygen evolution.[2] In the present investigation the process of carbon dioxide evolution during photosynthesis has been followed by measuring the release of $^{14}CO_2$ in light from leaf segments which had previously incorporated the isotope.

It was of interest to determine the source of the CO_2 evolved by green leaves in the light. Some of the evidence suggests that the production of CO_2 in the light is by a mechanism different to that which occurs in the dark. Firstly the rate of respiration which occurs in the light may differ from that which occurs in darkness for example, it has been shown that oxygen absorption by tobacco leaves in a CO_2-free atmosphere is stimulated by light.[1] Also $^{14}CO_2$ production by labelled rice leaves has been found to be stimulated by light.[3] Not only can the rate of respiration in light differ from that in darkness but there is also evidence that the two processes differ in their sensitivity to changes in external oxygen tension,[4] thus implying a different mechanism. The present investigation has yielded evidence

[1] N. P. VOSKRESENSKYA and G. S. GRISHINA, *Fiz. Rast.* **8**, 579 (1962).
[2] A. H. BROWN, *Am. J. Botany* **40**, 719 (1953).
[3] K. NISHIDA, *Plant Cell Physiol.* **3**, 111 (1962).
[4] G. KROTKOV, *Photosynthetic Mechanisms in Green Plants*, (Nat. Acad. Sci. Publ. No. 1145) 452. National Research Council, Washington (1963).

that respiration in the light uses a different substrate to that in darkness, and has, by the use of specific metabolic inhibitors, suggested a possible source for the CO_2 evolved in light.

RESULTS AND DISCUSSION

Segments of destarched tobacco leaves were allowed to photosynthesize in an air stream containing $^{14}CO_2$ for a standard pretreatment period. The subsequent release of total CO_2 and $^{14}CO_2$ was then examined under various conditions.

1. *On the Difference in Origin of the CO_2 Released by Leaf Segments in Light and in Darkness*

After pretreatment, the gas stream over the leaf segment was changed to CO_2-free air and the leaf chamber darkened. The specific activity of the CO_2 evolved by the segment under

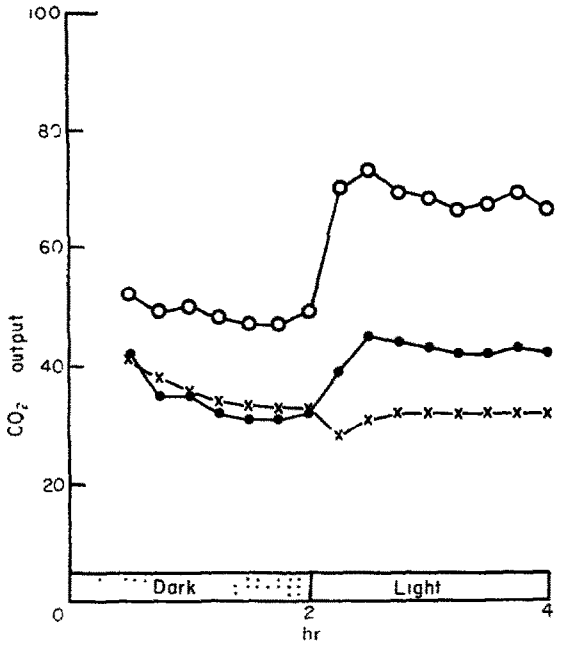

FIG. 1. THE RELEASE OF CO_2 INTO CO_2-FREE AIR BY A LABELLED TOBACCO LEAF SEGMENT IN THE LIGHT AND IN THE DARK.

×——× Total CO_2 release measured as ppm CO_2 concentration in the air leaving the segment.
●——● $^{14}CO_2$ release measured as counts evolved in 15 min per 10,000 originally taken up by the segment.
○——○ Specific activity of the CO_2 evolved measured as a percentage of the specific activity of the CO_2 fed during the pretreatment.

these conditions can be seen in Fig. 1, where it is compared with that of the CO_2 evolved when the segment was subsequently illuminated. The specific activity of the CO_2 evolved in the light was clearly much higher. This cannot be accounted for merely by a photosynthetic refixation of respiratory CO_2 with a discrimination against $^{14}CO_2$ because the increase in the specific activity which occurred in the light was accompanied by an increase in the absolute amount of $^{14}CO_2$ released by the segment. A more plausible explanation is that the CO_2 evolved in light comes from a different substrate to that involved in darkness, the higher

specific activity in light indicating that a greater use is made of recent assimilate under these conditions.

Figure 2 shows the result of a longer term experiment, in which a pretreated leaf segment was maintained in the light in a CO_2-free air stream for 16 hr. It can be seen that the segment released CO_2 at a constant rate and that the specific activity of the CO_2 was maintained at a very high level throughout. Since the specific activity was almost as high as that of the CO_2 fed during the pretreatment, we may conclude that it came largely from the assimilate of that period. Furthermore, during the 16 hr of the experiment, over 20 per cent of the total radioactivity taken up by the leaf had been lost again as CO_2, thus showing that a considerable proportion of the assimilate can be oxidized in this way.

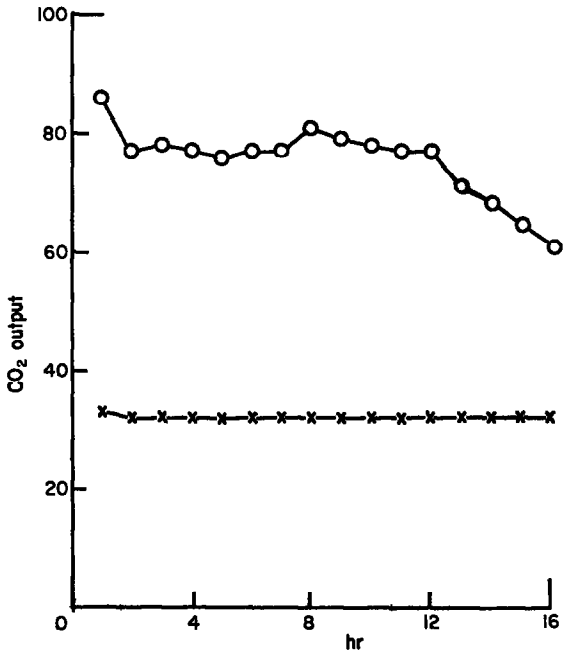

FIG. 2. THE RELEASE OF CO_2 INTO CO--FREE AIR BY A LABELLED TOBACCO LEAF SEGMENT IN THE LIGHT OVER A PROLONGED PERIOD.

×———× Total CO_2 release measured as ppm CO_2 concentration in the air leaving the segment.
o———o Specific activity of the CO_2 evolved measured as a percentage of the specific activity of the CO_2 fed during the pretreatment.

2. *On the Release of $^{14}CO_2$ into Air containing $^{12}CO_2$*

A $^{14}CO_2$ pretreated leaf segment was supplied in light with air containing $^{12}CO_2$ at atmospheric concentration (300 ppm). It was found that the release of $^{14}CO_2$ into such air was very much reduced when compared with that normally released into CO_2-free air, but when CO_2-free air was subsequently supplied, the rate of $^{14}CO_2$ release rose (see Fig. 3). The reason for this is not clear but it may be because the presence of external CO_2 inhibits CO_2 output in the light. However, it may also be because the presence of external CO_2 stimulates the refixation of respired CO_2, or alternatively, the CO_2 given off in light may be derived preferentially from new assimilate, and would therefore lose radioactivity when $^{12}CO_2$ was being fixed.

Figure 3 also shows the effect of a similar concentration of external $^{12}CO_2$ on the release of $^{14}CO_2$ from a pretreated leaf segment in the dark. It can be seen that in this case external CO_2 had no effect on the rate of $^{14}CO_2$ evolution.

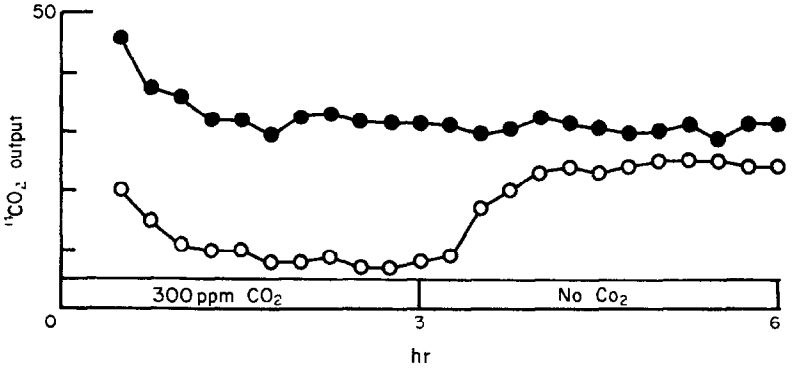

FIG. 3. THE RELEASE OF $^{14}CO_2$ INTO AIR CONTAINING $^{12}CO_2$ OR CO_2-FREE AIR BY A LABELLED TOBACCO LEAF SEGMENT, MEASURED AS COUNTS RELEASED IN 15 MIN PER 10,000 ORIGINALLY TAKEN UP BY THE SEGMENT.
— — — $^{14}CO_2$ released in the light.
● ——— ● $^{14}CO_2$ released in the dark.

3. *On the Mechanism of CO_2 Production in the Light*

From measurements of the CO_2 compensation point for the photosynthesis of tobacco leaves at different oxygen tensions Krotkov[4] deduced that CO_2 production in the light was stimulated by high oxygen concentration. As a means of obtaining direct confirmation of Krotkov's deduction, the effect of pure oxygen on the release of $^{14}CO_2$ from pretreated leaf segments was examined. A leaf segment was pretreated according to the standard procedure and then supplied with a stream of CO_2-free air for 3 hr followed by a stream of CO_2-free oxygen for a further 3 hr, the light remaining on for the whole time. Figure 4A shows that the effect of oxygen was to approximately double the total output of CO_2 in the light, this being accompanied by a parallel increase in $^{14}CO_2$ release. Figure 4B shows the result of a similar experiment in which the effect of oxygen on CO_2 released under CO_2-free conditions in darkness was studied. Here it can be seen that the stimulation of CO_2 and $^{14}CO_2$ production by oxygen was small compared with that which occurred in the light.

The fact that oxygen has differing effects on the rate of CO_2 output in the light and in the dark suggests that there may be two different mechanisms involved. The stimulation by oxygen of the rate of CO_2 production in the light gave a clue as to its possible origin. It has been shown in *Chlorella*[5] that high oxygen concentration stimulates the production of glycollate during photosynthesis. In addition, it has been shown by the use of inhibitors[6] that about 50 per cent of the carbon fixed photosynthetically by tobacco leaves passes through a glycollate stage. It seemed possible, therefore, that the release of CO_2 in the light from tobacco leaves resulted from the oxidation of glycollate. This hypothesis was tested by examining the effect of known inhibitors of glycollate metabolism on the evolution of $^{14}CO_2$ by pretreated leaf segments. The effect of the inhibitors on the specific activity of the CO_2 released was studied rather than on the total output of CO_2. The reason for this was

[5] J. COOMBS and C. P. WHITTINGHAM, *Phytochem.* **5**, 643 (1966).
[6] I. ZELITCH, *J. Biol. Chem.* **234**, 3077 (1959).

two-fold. Firstly, one of the inhibitors (α-hydroxy-2-pyridine-methane sulphonate) affects stomatal aperture.[7] This could in turn affect total CO_2 output but not its specific activity. Secondly, inhibition of glycollate oxidation may not inhibit total CO_2 production since the tissue, when denied the use of glycollate, may utilize another substrate in its place. If, however, the tissue responded to the inhibition of glycollate oxidation by going over to the mechanism of "dark respiration" then a reduction in the specific activity of the CO_2 released would be expected.

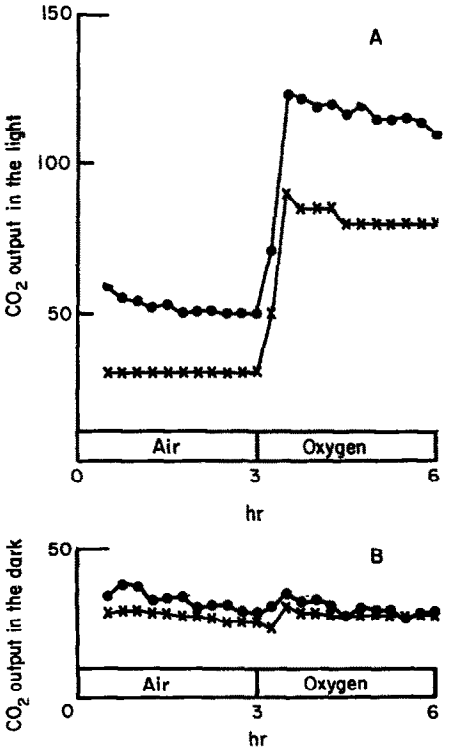

FIG. 4. THE RELEASE OF CO_2 INTO CO_2-FREE AIR AND CO_2-FREE OXYGEN BY A LABELLED TOBACCO LEAF SEGMENT, A IN THE LIGHT, B IN THE DARK.

×——× Total CO_2 release measured as ppm CO_2 concentration in the air leaving the segment.
●——● $^{14}CO_2$ release measured as counts evolved in 15 min per 10,000 originally taken up by the segment.

Two inhibitors of glycollate metabolism were tested, namely α-hydroxy-2-pyridine-methane sulphonate which inhibits its oxidation to glyoxylate by glycollic acid oxidase,[6] and iso-nicotinyl hydrazide which inhibits enzymes requiring pyridoxal phosphate and is believed to inhibit a later stage in glycollate metabolism, that is the enzymic conversion of glycine to serine.[8] Both inhibitors were used at a concentration of 10 mM.

In these experiments the normal pretreatment period was modified slightly. Instead of the leaf segment being floated on distilled water, it was floated on an equal volume of the inhibitor solution at pH 5·0. It was also maintained on this solution for the remainder of

[7] I. ZELITCH and D. A. WALKER, *Plant Physiol.* **39**, 856 (1964).
[8] G. G. PRITCHARD, C. P. WHITTINGHAM and W. J. GRIFFIN, *J. Exptl Botany* **14**, 281 (1963).

the experiment. The presence of the inhibitor had little effect on the amount of $^{14}CO_2$ taken up during pretreatment. After pretreatment the leaf segment was illuminated in CO_2-free air for 16 hr, then the light was extinguished for a further 4 hr. It can be seen from Fig. 5 that the specific activity of the CO_2 released in the light declined more rapidly in the presence of the inhibitors than it did in the absence of inhibitor. The onset of darkness had no effect on the specific activity of the CO_2 released by the inhibited segments. However the specific activity of the CO_2 evolved by the segment in the absence of inhibitor dropped sharply, when the light was extinguished, to approximately the level for that of the inhibited

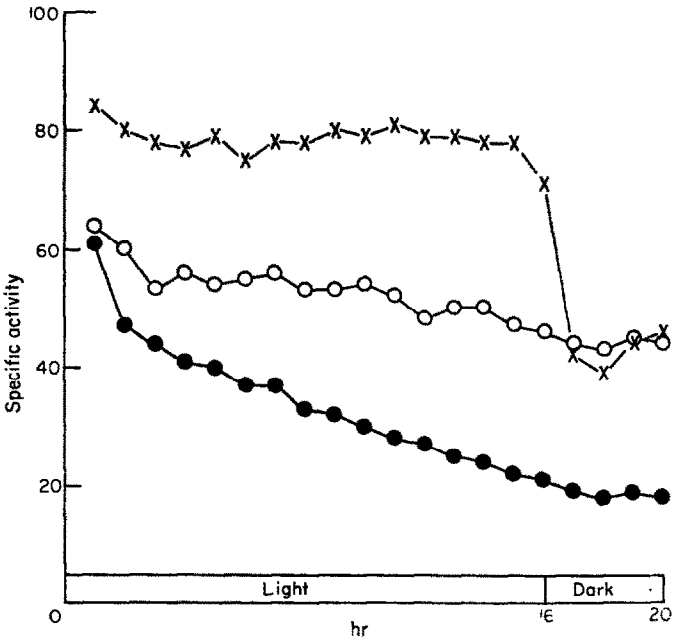

FIG. 5. THE EFFECT OF α-HYDROXY-2-PYRIDINE-METHANE SULPHONATE AND ISONICOTINYL HYDRAZIDE ON THE SPECIFIC ACTIVITY OF THE CO_2 RELEASED INTO CO_2-FREE AIR BY A LABELLED TOBACCO LEAF SEGMENT IN THE LIGHT.

The specific activity is measured as a percentage of the specific activity of the CO_2 fed during the pretreatment.

×——× No inhibitor.
○——○ 10 mM α-hydroxy-2-pyridine-methane sulphonate.
●——● 10 mM iso-nicotinyl hydrazide.

segments. This indicates that the stimulation of the release of $^{14}CO_2$ occasioned by light is inhibited by both α-hydroxy-2-pyridine-methane sulphonate and iso-nicotinyl hydrazide. Although it must be admitted that this evidence is not conclusive, because in this type of experiment the specificity of the inhibitor can never be beyond question, the results are consistent with the hypothesis that a considerable proportion of the CO_2 produced by a tobacco leaf in the light is derived from the metabolism of glycollate.

EXPERIMENTAL METHODS

The experimental material was derived from tobacco plants of the White Burley variety. Plants which had been grown in a greenhouse for several months were used. They were

transferred to a dark-room for 48 hr prior to use in order to deplete the starch content of the leaves. In each experiment a fully expanded leaf was removed and a 10×5 cm rectangular segment excised from one side of the midrib. The segment was floated, undersurface upwards, on 20 ml of distilled water in a rectangular perspex chamber whose total volume was 300 ml. An air stream, into which had been generated[9] a 300 ppm concentration of CO_2 was passed at a rate of 5 l./hr through the chamber, each litre containing 2 μc of $^{14}CO_2$. The leaf segment was illuminated from above at 40,000 lx using a 250 W tungsten spot-lamp. The leaf chamber was cooled by immersion 10 cm below the surface of a water bath at 20°. The air stream, on leaving the leaf chamber, was dried by passing it through a column of magnesium perchlorate. The $^{14}CO_2$ concentration was then assayed by passing the air through a cylindrical brass chamber of 15 ml volume, into the top of which had been sealed an end window G.M. tube. This was connected to an automatic scaler which printed out the total count at preset intervals after correcting this figure by subtracting the background count. The air stream was passed from the counting chamber to a "null balance" i.r. gas analyser for measurement of total CO_2 concentration. After passing through the analyser, the $^{14}CO_2$ was trapped in soda-lime and the gas stream discharged to waste. In all experiments this $^{14}CO_2$ assimilating period lasted for 6 hr, during which the $^{14}CO_2$ uptake was measured by noting the reduction in the radioactivity of the gas stream after it had left the leaf chamber. In all cases approximately two-thirds of the $^{14}CO_2$ supplied was taken up.

The above constituted the standard pretreatment of each leaf segment. The experiments, which have been described, differ in the subsequent treatment of the segments. However, the same light intensity was used as during the pretreatment, namely 40,000 lx. The gas flow rate was maintained at 5 l./hr so as to enable the count rate recorded for the $^{14}CO_2$ evolved by the segment to be compared directly with that for the $^{14}CO_2$ taken up during the pretreatment. The $^{14}CO_2$ output was measured over 15-min counting periods, the count rate recorded being a measure of the mean $^{14}CO_2$ concentration in the gas stream during the period. The results were finally expressed as the number of counts given out in each counting period per 10,000 originally taken up during the whole pretreatment. The count rate for the first 15 min after pretreatment was not used because of the presence of residual $^{14}CO_2$ from the pretreatment in the apparatus for part of this time. For measurements of the specific activity of the CO_2 evolved by the leaf segment, a stream of CO_2-free air was supplied. The count rate for the CO_2 evolved was then divided by the mean CO_2 concentration, as measured by the i.r. analyser during the period of counting. This gave a measure of the relative specific activity of the CO_2 evolved, which was finally expressed as a percentage of the specific activity of the CO_2 fed during pretreatment.

[9] A. GOLDSWORTHY, *J. Exptl Botany*, 17, 147 (1966).