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Glycolate Synthesis in Tomato Leaf Disks: Involvement of Storage Material in Photorespiration

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Glycolate accumulated in tomato leaf disks in the presence of α -hydroxy 2-pyridinemethanesulfonic acid during photosynthesis in the presence of $^{14}\text{CO}_2$. Based on the specific radioactivities of the glycolate and CO_2 , and the amount of ^{14}C in the glycolate pool, the amounts of glycolate and glycolate originating from CO_2 fixation were calculated. At 21 % O_2 , the glycolate accumulation increased with increasing CO_2 concentration to a maximum at 200-400 ppm but was inhibited when the CO_2 level was raised further. The accumulation of glycolate originating from CO_2 was reduced and the specific activity of glycolate relative to the added CO_2 decreased significantly at low CO_2 concentration. These findings suggest that at low CO_2 concentration, the main source of glycolate carbon is in the storage material. Glycolate formation and its carbon sources were further analyzed by varying the O_2/CO_2 ratio in the atmosphere, temperature and light intensity. The results suggested that glycolate is synthesized from storage material when the O_2/CO_2 ratio in the atmosphere is high and that decreased photosynthesis restricts the synthesis of glycolate originating from CO_2 .

INTRODUCTION

The glycolate pathway is responsible for photorespiration. The substrate of the pathway, glycolate, is synthesized mainly through oxygenation of RuBP (Andrews and Lorimer, 1978). O_2 competes with CO_2 at the catalytic site of RuBP carboxylase/oxygenase. Experiments with intact chloroplasts have revealed that a high O_2/CO_2 ratio favors glycolate synthesis (Ellyard and Gibbs, 1969; Robinson and Gibbs, 1974).

In intact leaves, however, determination of glycolate synthesis is difficult, because of the high activity of glycolate oxidase. Zelitch and Walker (1964) reported that glycolate accumulation in tobacco leaves decreased at high CO_2 concentrations in the presence of HPMS²⁾, an inhibitor of glycolate oxidase. Lee and Whittingham (1974) also found that $^{14}\text{CO}_2$ incorporation into the glycolate pathway decreased at high CO_2 concentrations. On the other hand,

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1) RuBP, ribulose-1, 5-bisphosphate

2) HPMS, α -hydroxy 2-pyridinemethanesulfonic acid

Bravdo and Canvin (1979) reported that rates of photorespiration decreased little at high CO_2 concentrations. Yamauchi and Yamada (1981) suggested that the $^{14}\text{CO}_2$ incorporation into the glycolate pathway, as measured by ^{14}C entering the serine pool, was substantial at high CO_2 concentrations. The effect of CO_2 concentration on the glycolate pathway thus remains unclear.

When $^{14}\text{CO}_2$ was applied to illuminated leaves, the specific radioactivity of glycolate was about 50 to 100 % of the $^{14}\text{CO}_2$ (Zelitch, 1975; Agrawal and Fock, 1978), suggesting that the carbon of CO_2 fixed during photosynthesis is a principal source of glycolate carbon. However, Ludwig and Canvin (1971) and Fock et al. (1979) reported that the specific activity of photorespired CO , decreased at lower CO_2 concentrations. The amount of $^{14}\text{CO}_2$ incorporated into the glycolate pathway was found by us to decrease at lower CO_2 concentrations (Yamauchi and Yamada, 1981), where substantial glycolate is formed (Oliver, 1979). It is proposed that storage material is involved in photorespiration at low CO_2 concentrations (Mahon et al., 1974 a; Beck, 1979, Yamauchi and Yamada, 1981, 1982).

In the present study, glycolate was accumulated in tomato leaf disks by blocking its oxidation with HPMS (Zelitch, 1973) during photosynthesis in $^{14}\text{CO}_2$. The amounts of glycolate and glycolate whose carbon source was in photosynthetically fixed CO , were calculated on the basis of the specific activities of glycolate and CO , and of the amount of ^{14}C in the glycolate pool. Furthermore, the specific activity of glycolate was compared with that of CO , in order to evaluate the photosynthetically fixed carbon in glycolate synthesis. It is suggested that storage material is involved in glycolate synthesis in an atmosphere of high O_2/CO_2 ratio.

MATERIALS AND METHODS

Plant materials

Tomato plants (*Lycopersicon esculentum* Mill cv. Fukuju No. 2) were grown under natural light in solution culture (Yamauchi and Yamada, 1980). NaNO_3 was used as the nitrogen source. Young fully-developed 6th or 7th leaves (6 to 8 weeks old) were detached under water and stored with their cut bases in water at room temperature (20–25°C) in the dark until use (less than 12 h).

Gas exchange apparatus and techniques of glycolate accumulation

After oxygen and/or nitrogen gas had been admitted into a Tedlar bag (Du Pont Co., Ltd.) of 20 or 100 liters, the desired proportion of CO , or $^{14}\text{CO}_2$ was introduced into the bag. The CO , or $^{14}\text{CO}_2$ had been prepared by adding lactic acid to Na_2CO_3 or $\text{Na}_2^{14}\text{CO}_3$. The gas mixture was prepared just before use. The ^{14}C in a unit volume of the $^{14}\text{CO}_2$ -gas mixture was determined by liquid scintillation counting after dissolving the $^{14}\text{CO}_2$ in an ethanolamine-methyl cellosolve mixture (1: 3, v/v). The concentration of CO_2 was measured with an infrared CO_2 analyzer. The specific radioactivity of $^{14}\text{CO}_2$ was calculated from the ratio of two values.

Glycolate was accumulated by the method of Zelitch (1973) and Oliver (1979). Nine disks (1.5 cm in diameter, about 0.245 g fr wt) per sample were punched from the detached leaves, strung together on a thread and placed in the bottom of a 100-ml Erlenmeyer flask. The disks were then washed with 5 ml of water 2 to 3 times and floated on 5 ml of water. The flask was placed in an illuminated water bath at a prescribed temperature (see figure legends). A rubber stopper with two glass tubes and two long hollow needles was used to stopper the flask. Gas was exchanged through the glass tubes. The hollow needles, attachable to syringes, were used to remove the water and to add 10 mM HPMS solution, on which the disks floated.

The gas mixture of $^{14}\text{CO}_2$ with the desired composition of O_2 , N_2 and CO , was passed through the flask at a flow rate of 200 ml min^{-1} after flowing a $^{12}\text{CO}_2$ gas mixture of the same composition for 30 min. APS³⁾ was determined by measuring the decrease in $^{14}\text{CO}_2$ concentration and was stable during the $^{14}\text{CO}_2$ application. After application of the $^{14}\text{CO}_2$ for 30 min, the water in the flask was removed and replaced with 5 ml of 10 mM HPMS through the needle. The flask was then shaken gently once. The flow of the $^{14}\text{CO}_2$ -gas was stopped and the flask was opened 170 s after the injection of HPMS. The disks were removed at 180 s using the thread and killed in 15 ml of boiling 20 % ethanol.

Preliminary tests (Yamauchi, 1982) revealed that glycolate accumulated linearly for at least 180 s after the application of HPMS. The specific activity of glycolate accumulated following HPMS application increased after starting photosynthesis in $^{14}\text{CO}_2$ and reached a stable value before 30 min.

Analysis of the concentration and specific activity of glycolate

The killed disks were homogenized in 20% ethanol with a glass homogenizer. The homogenate was filtered through a glass filter with the aid of hyflo super-cell under reduced pressure. The filtrate was passed through a Dowex 1 x8 (200-400 mesh) acetate column (1. Ox 10 cm). Glycolate was eluted from the column with 50 ml of 4 M acetic acid (glycolate fraction). This contained glycolate, glycerate and unknown compounds which can hinder color development in Calkins' test (Calkins, 1943). The glycolate concentration in the glycolate fraction was therefore not obtained directly by the test. It was calculated on the basis of the amount of ^{14}C (dpm) in and the specific activity (dpm/ μmol) of glycolate, which were measured as described below.

The glycolate fraction was concentrated under reduced pressure at 40°C to 1 ml. The recovery of glycolate in the concentrated fraction, which was measured by adding a known amount of $1\text{-}^{14}\text{C}$ -glycolate to the killed leaf disks, was about 95%.

The amount of ^{14}C in the glycolate in the glycolate fraction was measured as follows. A portion of the concentrated glycolate fraction was spotted on Whatman 3 MM paper together with carrier glycolic acid (25–50 μl of 10 mg/ml aqueous solution) because glycolate is volatile. It was developed by ascending

3) APS, apparent photosynthesis

chromatography for 18 h at room temperature with ethylether/acetic acid/water (15: 3: 1, v/v). After development, the chromatograms were dried at room temperature, and the glycolic acid was located by spraying with 0.04 % bromocresol green (pH 7.0). The glycolate was eluted from the paper with water and its ^{14}C was determined by liquid scintillation counting. Above 95 % of ^{14}C -glycolate, which had been spotted on the paper with the carrier, was recovered by this procedure.

The amount of glycolate formed from CO , (namely $^{14}\text{CO}_2$ and $^{12}\text{CO}_2$) was calculated on the basis of the amount of ^{14}C (dpm) in the glycolate and the specific activity (dpm/ μmol) of the $^{14}\text{CO}_2$ applied.

The specific activity of glycolate was determined as follows: The concentrated glycolate fraction from the Dowex 1 column was subjected to paper chromatography without carrier to remove the hindering substances of Calkins' test. ^{14}C -glycolate was eluted with water from the chromatogram, and its concentration was then determined by Calkins' test and its ^{14}C by liquid scintillation counting. The specific activity of glycolate was calculated from these two values.

Preliminary tests confirmed the accuracy of values for the specific activity of glycolate obtained by this method. When $0.26 \mu\text{mol}$ of glycolic acid ($2.50 \sim 10^5$ dpm μmol^{-1}) was chromatographed, its specific activity was lowered to 85 % of the initial value, as an average of two determinations. On the other hand, the specific activity of glycolate in different volumes of tomato leaf extract was determined after adding a constant amount of ^{14}C glycolic acid (Fig. 1). This control test revealed that the reciprocal of the specific activity of glycolate varied linearly over the range of one-fifth to two times the amount of glycolate accumulated per sample in normal air. This indicated that the method used to determine the specific activity was valid for comparisons over this range of glycolate, although there was an error in the absolute values of the specific activity. When a serious error was expected in measuring the specific activity of glycolate, it will be specified in the text.

RESULTS AND DISCUSSION

The relative specific activity (RSA) of glycolate was expressed as:

$$\frac{\text{Specific activity of glycolate}}{\text{Specific activity of CO, applied}} \times 100 (\%)$$

When glycolate is synthesized from photosynthetically fixed CO_2 , RSA becomes 100 %. A decrease in RSA means that part of the glycolate originates from a source other than the fixed CO_2 .

The glycolate accumulation rate in the presence of HPMS at 21% O_2 was about $40 \mu\text{mol g fr wt}^{-1} \text{h}^{-1}$ (calculated from Fig. 2). The glycolate synthesis in the tomato leaf disks was also evaluated from the CO efflux into CO_2 -free air. The CO_2 efflux rate was found to be about $31 \mu\text{mol g fr wt}^{-1} \text{h}^{-1}$ in the light and about $19 \mu\text{mol g fr wt}^{-1} \text{h}^{-1}$ in the dark. If one assumes that all the

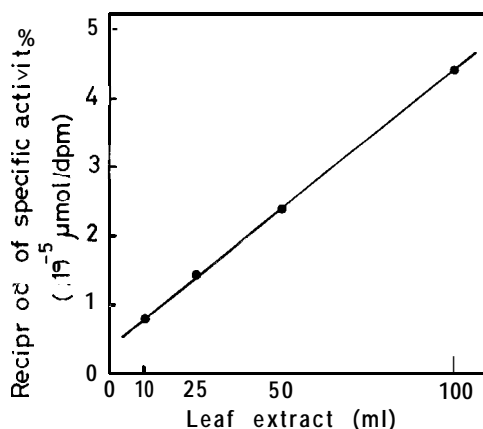


Fig. 1. Control tests of the method for determining the specific activity of glycolate.

$1\text{-}^{14}\text{C}$ -glycolic acid ($0.109 \mu\text{mol}$, $2.07 \times 10^{-5} \text{ dpm } \mu\text{mol}^{-1}$) was added to a tomato leaf extract of 10, 25, 50 or 100 ml. The specific activity of glycolate in each extract was then determined by the method described under Materials and Methods. The tomato extract was from leaf disks treated in air for 180 s with HPMS. Fifty ml of the leaf extract corresponded to one sample analysis in other experiments. The ordinate represents the reciprocal of the specific activity.

CO_2 , released in the light originates from the glycolate pathway and that 2 glycolates from 1 CO_2 , and serine (Tolbert, 1971), the glycolate synthesis could be as high as $62 \mu\text{mol g fr wt}^{-1}\text{h}^{-1}$. If it is assumed that the CO_2 , released in the light contained dark respiration, then the CO_2 , release from photorespiration would be $12 \mu\text{mol g fr wt}^{-1}\text{h}^{-1}$. The glycolate accumulation rate is within the range estimated from the CO_2 efflux.

The extent of HPMS penetration into the tissue is obscure in our method of glycolate accumulation. Moreover, HPMS inhibits enzymes other than glycolate oxidase and reduces the photosynthesis rate (Liittge *et al.*, 1972). However, the glycolate accumulation rate is sufficient to explain the degree of photorespiration, which is obtained only when HPMS is applied to the leaf tissue (Zelitch, 1979). Although the detailed mechanism of glycolate accumulation in HPMS-treated leaf disks has not yet been clarified, this method could be useful in glycolate synthesis studies on intact leaf tissues.

At 21% O_2 , APS increased with increasing CO_2 concentration up to 1000 ppm (Fig. 2). The RSA of glycolate was maximum (92-94 %) at 200-500 ppm CO_2 , and minimum at low CO_2 concentration. The RSA decreased with increasing CO_2 concentrations above 525 ppm. The effect of CO_2 concentration on the RSA of glycolate was consistent with those of glycine and serine, intermediates in the glycolate pathway, reported by Mahon *et al.* (1974 a). The striking decrease in RSA of glycolate at low CO_2 concentrations indicates that there is a glycolate carbon source other than photosynthesis.

Glycolate accumulation increased with increasing CO_2 concentration to a

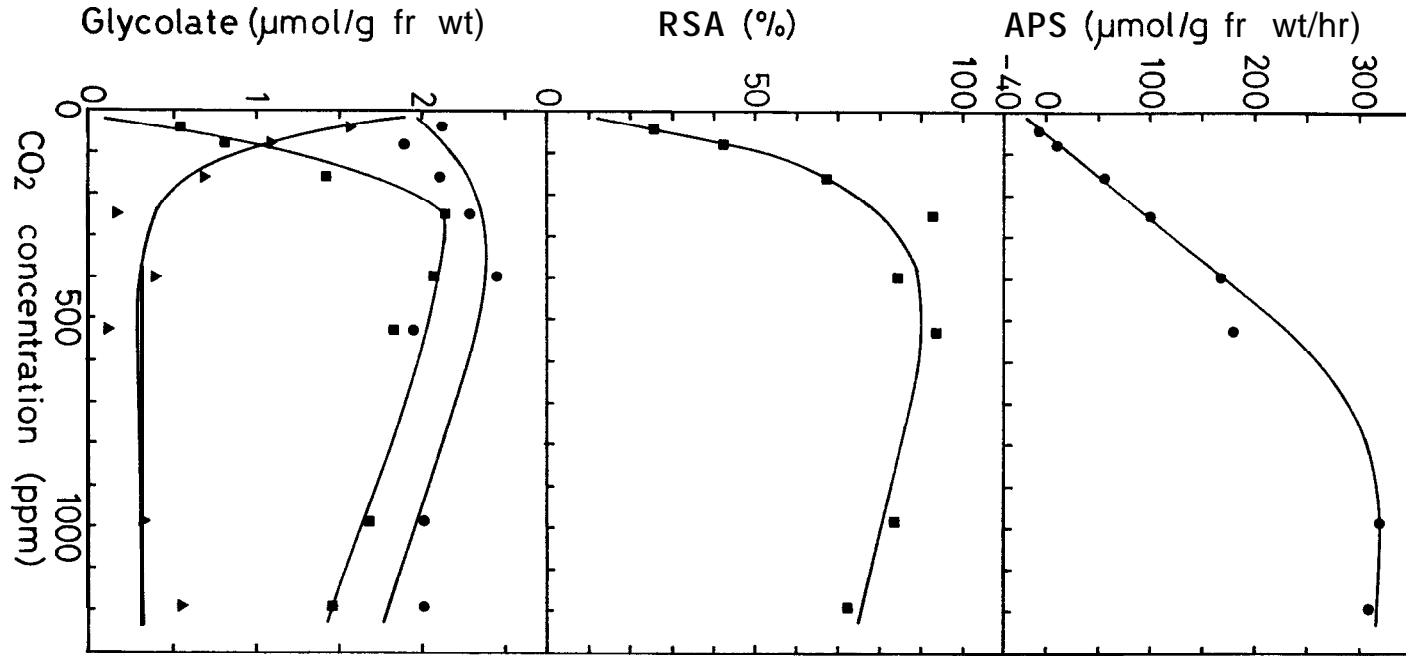


Fig. 2. Effects of CO_2 concentration on APS, RSA of glycolate and its accumulation.

In the lower graph, \blacktriangle indicates the difference in accumulation of glycolate (\bullet) and glycolate whose carbon was from photosynthetically fixed CO_2 (\blacksquare). A flask with 9 disks was immersed in a water bath at 25°C . The photon flux density at the surface of the disks was $600\text{--}660\ \mu\text{E m}^{-2}\text{s}^{-1}$. The disks in $21\ \%$ O_2 were maintained in CO_2 for 30 min and in $^{14}\text{CO}_2$ at the same concentration for the following 30 min. Then, 10 mM HPMS was applied to the disks for 180 s. The concentrations (ppm) and specific activities of $^{14}\text{CO}_2$ ($\text{dpm}\ \mu\text{mol}^{-1}$) applied to the disks were as follows: 44, 1.94×10^5 ; 78, 1.30×10^5 ; 158, 7.37×10^5 ; 249, 7.63×10^5 ; 396, 6.09×10^5 ; 525, 4.70×10^5 ; 985, 5.41×10^5 ; and 1190, 3.84×10^5 , respectively.

maximum at 200-400 ppm CO₂, and was then slightly reduced at higher CO₂ levels (Fig. 2). The glycolate accumulation from CO₂ was greatly lowered with decreasing CO₂ concentrations below 249 ppm, but its ratio to APS increased with such decreasing CO₂ concentration. The increased differences in accumulations of glycolate from CO₂ at lower CO₂ concentrations *also* indicate that there is a glycolate carbon source other than photosynthesis. The storage material represents a probable carbon source for glycolate synthesis. In the following discussion, therefore, we assume that glycolate carbon source other than photosynthetically fixed CO₂ was the storage material, and investigate its involvement in glycolate synthesis under various conditions.

APS decreased linearly with increasing O₂ concentration in the range of 435 to 507 ppm CO₂ (Fig. 3). The RSA of glycolate increased to a maximum between 25 and 50% O₂ and then decreased with increasing O₂ concentrations. The decrease in the RSA of glycolate when the O₂ concentration was lowered to 1% agrees with those of glycine and serine in sunflower leaves (Mahon *et al.*, 1974 b). The amount of glycolate accumulated at 0 % O₂, being 0.259 $\mu\text{mol g fr wt}^{-1}$, was too small for accurate measurement of its specific activity. The amount of glycolate from CO₂ decreased with increasing O₂ concentration above 25 %, but its ratio to APS increased. The glycolate from storage material increased with increasing O₂ concentration.

Fig. 4 shows the effects of O₂ concentration on APS and glycolate accumulation as a function of CO₂ concentration. The difference in glycolate accumulation from CO₂ between 1 % and 80 % O₂ was negligible at a low CO₂ concentration, but increased at high CO₂ concentrations. Storage material was involved in the glycolate accumulation at the lower CO₂ concentration and 80 % O₂, but not at 1 % O₂.

The effects of temperature on APS, RSA, and accumulation of glycolate were examined at 386 ppm CO₂ and 21% O₂ (Fig. 5). APS was maximum between 25 and 35°C. The RSA of the glycolate was not appreciably influenced by temperature over the range of 15 to 30°C, being 74 to 81%. This is consistent with the report of Fock *et al.* (1979) that the RSA of photoperiod CO₂ is independent of leaf temperature in the range of 18 to 30°C. Glycolate accumulation from storage material was slightly increased between 25 and 39°C. Glycolate accumulation was maximum between 25 and 35°C as well as APS.

The effect of light intensity on APS, RSA, and accumulation of glycolate was also examined at 450 ppm CO₂ and 21 % O₂ (Fig. 6). The glycolate accumulation increased with increasing light intensity as well as APS. Increasing light did not increase the glycolate accumulation from storage material at an intensity of up to 963 $\mu\text{E m}^{-2}\text{s}^{-1}$. The RSA of glycolate was constant except at an extremely low light intensity.

It has been established that glycolate synthesis in intact chloroplast is regulated by the ratio of O₂/CO₂ (Ellyard and Gibbs, 1969; Robinson and Gibbs, 1974). In tomato leaf disks, glycolate synthesis was decreased at high CO₂ and/or low O₂ concentrations (Figs. 2-4), which is consistent with studies using organellae. However, the glycolate synthesis from photosynthetically fixed CO₂ was decreased at low CO₂ and/or high O₂ concentrations (Figs. 2-4),

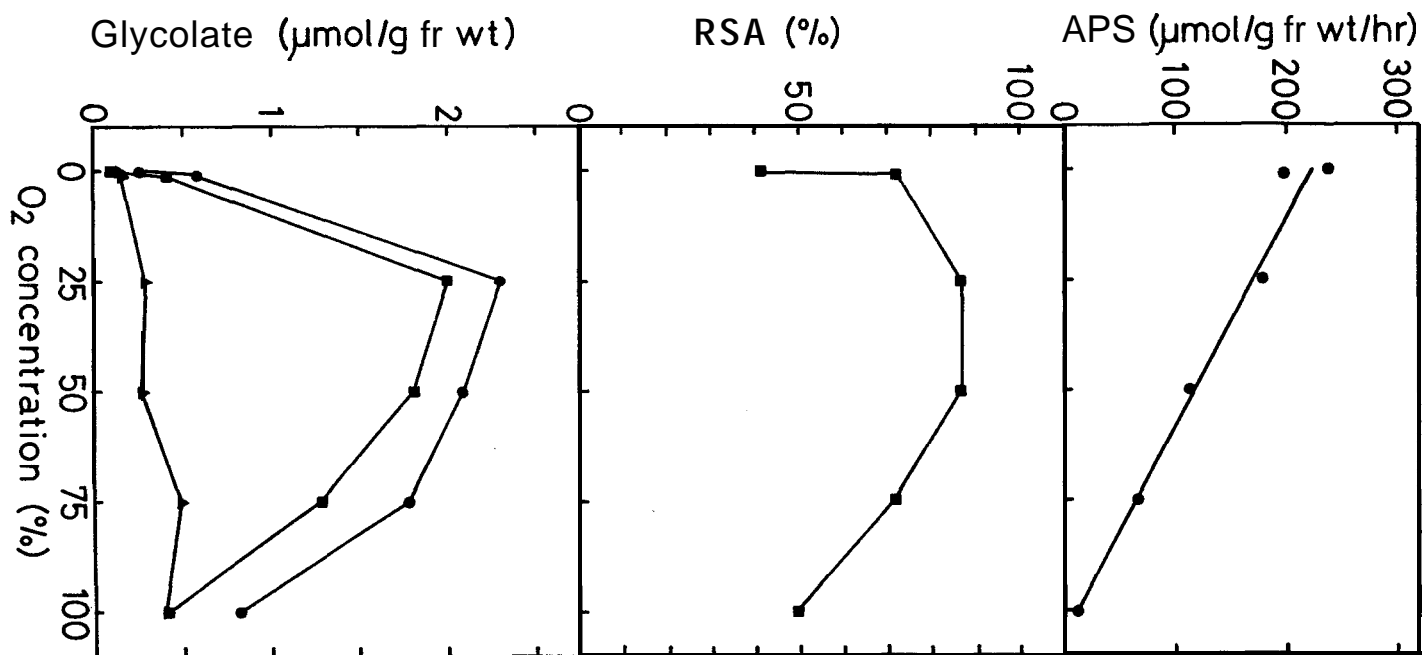


Fig. 3. Effects of O_2 concentration on APS, RSA of glycolate and its accumulation in tomato leaf disks.

▲ indicates the difference in accumulations of glycolate (●) and glycolate whose carbon was from photosynthetically fixed CO_2 (■). A flask with 9 disks was immersed in a water bath at $29^\circ C$. The photon flux density at the surface of the disks was $600-660 \mu E m^{-2} s^{-1}$. Before the application of $^{14}CO_2$ at the desired O_2 concentration, the disks were maintained in $^{12}CO_2$ at the respective O_2 concentration for 30 min. After 30 min photosynthesis in $^{14}CO_2$, the disks were treated with HPMS for 180 s. The concentration of O_2 (%) and CO_2 (ppm) and the specific activities of the latter (dpm μmol^{-1}) were as follows: 0, 478, 4.75×10^5 ; 1, 467, 4.82×10^5 ; 25, 472, 5.10×10^5 ; 50, 507, 5.12×10^5 ; 75, 476, 5.02×10^5 ; and 100, 435, 5.25×10^5 , respectively.

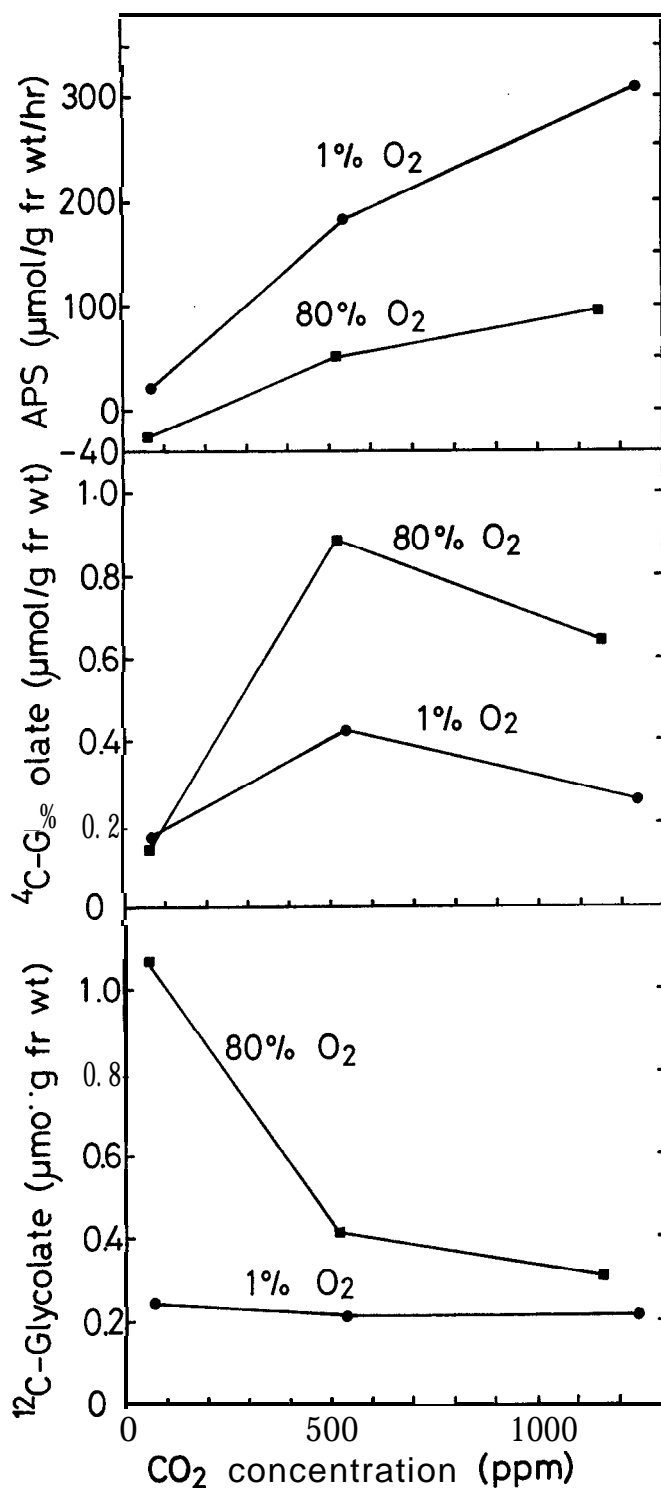


Fig. 4. Effects of CO₂ concentration on APS and glycolate accumulation in tomato leaf disks at 1% and 80% O₂. Glycolate whose carbon was from photosynthetically fixed CO₂ is presented in the middle graph. The lower graph shows the difference in accumulation of glycolate and glycolate from CO₂. A flask with 9 disks was immersed in a water bath at 24-25°C. The photon flux density of the surface of the disks was 600-660 μE m⁻² s⁻¹. Before the application of ¹⁴CO₂ at the desired CO₂ and O₂ concentrations, the disks were maintained in the same CO₂ and O₂ concentrations for 30 min. After 30 min photosynthesis in ¹⁴CO₂, the disks were treated with HPMS for 180 s. The concentrations of O₂ (%) and CO₂ (ppm) and the specific activities of the latter (dpm μmol⁻¹) were as follows: 1, 67, 1.80 × 10⁶; 1, 532, 4.72 × 10⁵; 1, 1235, 4.42 × 10⁵; 80, 59, 2.01 × 10⁶; 80, 512, 5.12 × 10⁵; and 80, 1148, 4.40 × 10⁵, respectively. The values of the RSA of glycolate at 1% O₂ were 40.8, 66.3 and 54.3% at 67, 532 and 1235 ppm CO₂ and those at 80% O₂ were 11.6, 68.2 and 68.0% at 59, 512 and 1148 CO₂, respectively.

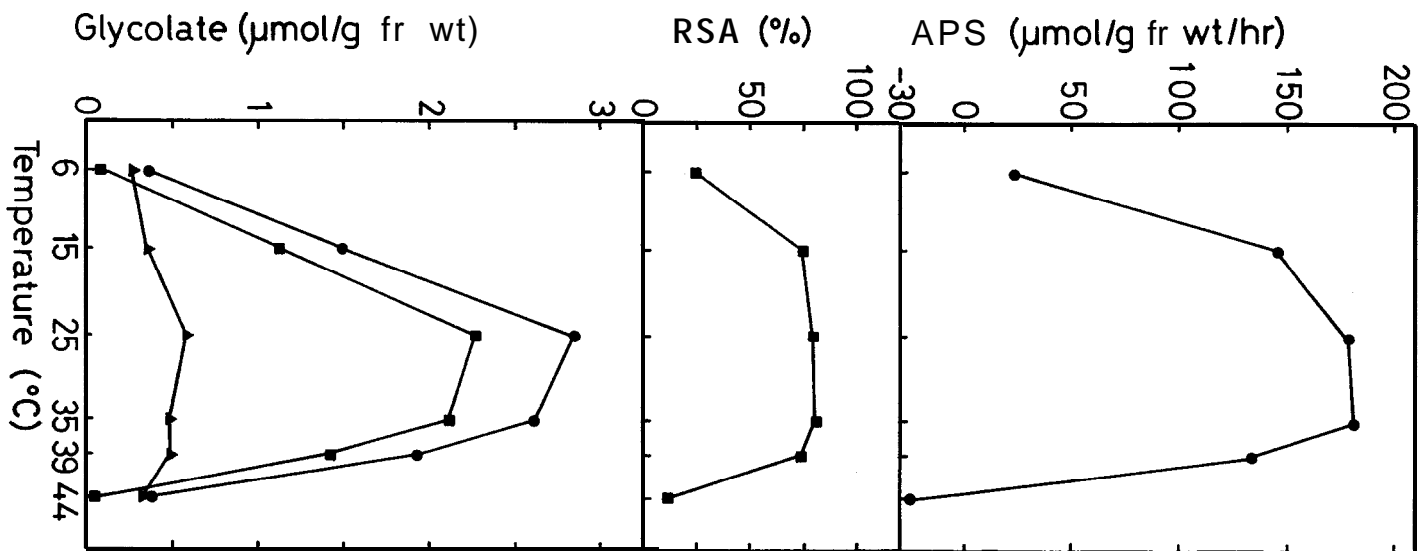


Fig. 5. Effects of temperature on APS, RSA of glycolate and its accumulation in tomato leaf disks.

▲ indicates the difference in accumulations of glycolate (●) and glycolate whose carbon was from photosynthetically fixed CO₂ (■). A flask with 9 disks was immersed in a water bath at the indicated temperatures. The photon flux density at the surface of the disks was about 470 $\mu\text{E m}^{-2}\text{s}^{-1}$. Before application of ¹⁴CO₂ at each respective temperature, the disks were maintained in ¹²CO₂ at the same temperature for 30 min. After 30 min photosynthesis in ¹⁴CO₂, the disks were treated with HPMS for 180 s. The concentration of ¹⁴CO₂ applied was 386 ppm and its specific activity was 2.19×10^5 dpm μmol^{-1} .

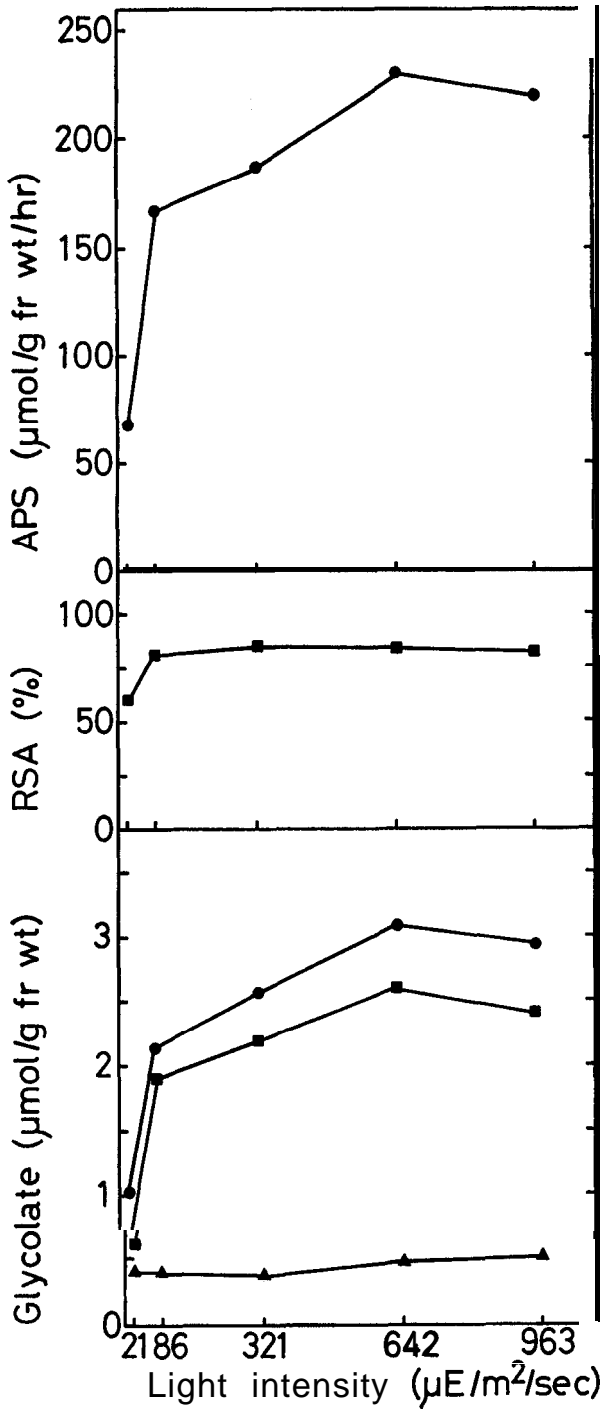


Fig. 6. Effects of light intensity on APS, RSA of glycolate and its accumulation in tomato leaf disks.

▲ indicates the difference in accumulations of glycolate (●) and glycolate whose carbon was from photosynthetically fixed CO_2 (■). A flask with 9 disks was immersed in a water bath at 25°C . Before application of $^{14}\text{CO}_2$ at each respective light intensity, the disks were maintained in $^{12}\text{CO}_2$ at the same light intensity for 30 min. After 30 min photosynthesis in $^{14}\text{CO}_2$, the disks were treated with HPMS for 180 s. The concentration of $^{14}\text{CO}_2$ applied was 450 ppm and its specific activity was $2.58 \times 10^5 \text{ dpm } \mu\text{mol}^{-1}$.

which contradicts the conclusions obtained from organellae. Decreased APS under those conditions could restrict the glycolate synthesis whose carbon is in photosynthesis.

Glycolate synthesis at low CO₂ concentrations occurred from storage material at 21 % and 80 % O₂ (Figs. 2 and 4). In the presence of more than 300 ppm CO₂, storage material was not used appreciably for glycolate synthesis even when the O₂ concentration was 100 % (Fig. 3). These phenomena could be explained by the ratio of O₂/CO₂ in the tissue. When APS is close to zero, the concentration of CO₂ in the leaf tissue would be equal to that surrounding the leaf. The ratios of O₂/CO₂ in the tissue became 4200 at 21% O₂ and 50 ppm (0.005 %) CO₂ (Fig. 2) and 4000 at 80 % O₂ and 200 ppm (0.02 %) CO₂ (Fig. 4), where storage material was involved in the glycolate synthesis. On the other hand, the ratio was 2300 at 100 % O₂ and 430 ppm (0.043 %) CO₂ (Fig. 3), when less storage material was used. Thus, storage material can be involved in glycolate synthesis as the ratio of O₂/CO₂ increases, compensating the decreased glycolate synthesis from photosynthetically fixed carbon.

Even when the external CO₂ supply is limited by environmental conditions, such as water-stress causing stomatal closure, photorespiration may continue in illuminated leaves using storage material as a carbon source for glycolate synthesis. Photorespiratory CO₂ evolved in the leaf under such conditions would protect the tissue against photoinhibition (Powles *et al.*, 1979) and/or photooxidative damage (Asami and Akazawa, 1978) through the dissipation of excess photochemical energy.

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