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THE PATH OF CARBON IN PHOTOSYNTHESIS. IX. PHOTOSYNTHESIS, PHOTO-REDUCTION AND THE HYDROGEN-OXYGEN-CARBON DIOXIDE DARK REACTION

Elmer J. Badin and Melvin Calvin

February 1, 1950

Berkeley, California

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by

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University of California, Berkeley

ABSTRACT

February 1, 1950

1.- A comparison of the rates of fixation of Carbon 14 dioxide in algae for the processes of photosynthesis, photoreduction and the hydrogen-oxygen-carbon dioxide dark reaction has been made.

2.- For the same series of experiments, rates of incorporation of tracer carbon into the separate soluble components using the radiogram method have been determined.

3.- The mechanism of carbon dioxide uptake has been shown to occur via two distinct paths.

4.- In all cases studied, essentially the same compounds appear radio-active. The distribution with time, however, differs markedly.

THE PATH OF CARBON IN PHOTOSYNTHESIS. IX. PHOTOSYNTHESIS, PHOTOREDUCTION AND THE HYDROGEN-OXYGEN-CARBON DIOXIDE DARK REACTION (1)

by

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It has been shown that certain algae after being allowed to remain in an atmosphere of molecular hydrogen in the dark acquire the ability to reduce carbon dioxide by two processes in addition to the normal reaction of photosynthesis represented by:

$$H_2O + CO_2 \quad \xrightarrow{\quad h\nu \quad} \quad (CH_2O) + O_2 \qquad (1)$$

These two additional reactions were first observed by Gaffron (3,4) and are

(1) Photoreduction (a light induced reaction occurring at low light intensity)

$$2H_2 + CO_2 \quad \xrightarrow{\quad h\nu \quad} \quad (CH_2O) + H_2O \qquad (2)$$

No oxygen is evolved in this reaction.

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(3) H. Gaffron, Nature, 143, 204 (1939).

(4) H. Gaffron, Science, 91, 529 (1940).

(2)   The hydrogen-oxygen-carbon dioxide reaction (a dark reaction)

$$2H_2 + O_2 + xCO_2 \longrightarrow \text{products} \qquad (3)$$

This latter process is presumed to occur as a result of, or be initiated by,

the hydrogen-oxygen reaction

$$2H_2 + O_2 \longrightarrow 2H_2O \qquad (4)$$
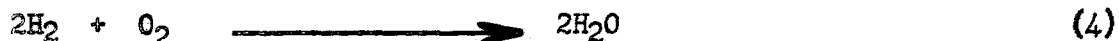
The ratio of the amount of carbon dioxide reacting to the amount of oxygen present in
reaction (3) has not been determined with certainty.

In this paper rate studies on the appearance of Carbon 14 in intermediates

and products are reported for (a) photosynthesis at low light intensity; (b) photo-

reduction at low light intensity; (c) the hydrogen-oxygen-carbon dioxide dark re-

action; and (d) the hydrogen-carbon dioxide dark reaction (same as (c) except ab-

sence of oxygen). The method used for separation and identification of products

has been the radiogram method as developed by Calvin and co-workers (5). The object

of the present work has been to further elucidate the mechanisms of the various

reactions, particularly in regard to the mechanism of carbon dioxide fixation.

EXPERIMENTAL

Experiments were carried out in a Warburg-type apparatus. All conditions

were identical (temperature, 20.7° C; moderate speed of shaking; 0.10 cc. centri-

fuged Scenedesmus D_3 in 2.9 cc. of 0.05 $\underline{M}$ KH_2PO_4 (pH~4.5) solution) except for the

variables noted later. The cell suspension was placed in the flask having a side

tube and attached to a manometer. A solution of $Na_2C^{14}O_3$ was placed in the side

(5)   A. A. Benson, J. A. Bassham, M. Calvin, T. C. Goodale, V. A. Haas and
       W. Stepka, J. Am. Chem. Soc., (in press).

arm tube. In all experiments except normal photosynthesis the flasks were flushed with hydrogen for one hour and allowed to remain in an atmosphere of hydrogen in the dark for 16-18 hours. At the end of this adaption time (a) the $Na_2C^{14}O_3$ was added from the side arm tube for the hydrogen-carbon dioxide dark experiments; (b) oxygen was added to a pressure corresponding to 0.5% of the total gas pressure for the hydrogen-oxygen-carbon dioxide dark reaction and the $Na_2C^{14}O_3$ added after the hydrogen-oxygen reaction had started; or (c) the light was turned on and the photoreduction process allowed to proceed until the pressure had leveled off, after which the $Na_2C^{14}O_3$ was added. For photosynthesis experiments, algae suspensions were shaken in the light (air atmosphere) for one hour after which $Na_2C^{14}O_3$ was added.

Six duplicate experiments were carried out simultaneously. At the end of a given time the entire contents of a flask were centrifuged in the dark, the supernatant liquid discarded, and an 80% boiling ethanol solution added to the algae. The time elapsing between sampling and water-alcohol extraction was 3-4 minutes. Experiments were carried out up to 300 minutes. The total radioactivity fixed was determined by direct counting on a thin plate of an aliquot of the alcohol slurry. The slurry was then centrifuged and an aliquot of the clear soluble component counted in the same manner. The soluble component was evaporated to dryness and a measured quantity of 50% ethanol-50% water solution added. An aliquot of this was chromatographed using two-dimensional filter paper chromatography. Solvents used were water-phenol and butanol-water-propionic acid (5). Each radioactive compound present on the paper was counted directly on the paper after its position was determined by a radioautograph.

The light intensity used for photosynthesis and photoreduction was about 790 ergs/cm^2/sec. ($\sim$45 foot candles).

A total quantity of $Na_2C^{14}O_3$ equivalent to 10.5 microcuries (about 3.1×10^6 cpm (counts per minute)) was used in each experiment. Upon introduction of this to the acid suspension of algae, carbon dioxide was evolved. Pressure-time data for the experiments showed in each case that the proper reaction was occurring.

RESULTS

The rates of fixation of tracer carbon for the four cases studied are shown in Figures 1, 2, 3 and 4. The relative rates of incorporation of tracer carbon in the components of the soluble fraction are shown in Figures 5, 6, 7 and 8. These two sets of data may be used as a basis for comparison of the four different processes investigated. Marked differences are shown for total carbon dioxide fixation (Figures 1 to 4). Significant differences are also shown for the rates of fixation of carbon dioxide into the various components (Figures 5 to 8). However, in the latter set of data it is seen that radioactivity is distributed among the same compounds whether in photoinduced reactions or dark reactions. Similarities and differences occur only in the rates of appearance of radioactivity in these compounds.

Relative slopes for carbon dioxide fixation are 5000, 9000, 900 and 150 cpm// 0.10 cc. cells/min. for photosynthesis, photoreduction, the hydrogen-oxygen-carbon dioxide dark reaction and the hydrogen-carbon dioxide dark reactions, respectively. The total radioactivity fixed in photoreduction was greater than in photosynthesis by a factor which appears to be greater than the experimental error. This might conceivably be due to a shift in internal pH of the algae during the hydrogen adaptation and photoreduction process. A significant difference between the light reactions (Figures 1 and 2) and the dark reactions (Figures 3 and 4) lies in the

relative absence of insoluble radioactive products in the latter cases. These products include, among others, proteins and cellulose. Another significant difference is observed between the two dark reactions (Figures 3 and 4). All other conditions being equal, addition of 0.5% oxygen is seen to increase the amount of carbon dioxide fixed by approximately a factor of six.

The second set of data (Figures 5, 6, 7 and 8) show the relative rates of incorporation of tracer carbon in the components of the soluble extract. The compounds have been grouped for plotting as organic phosphates (including phosphoglyceric acid and hexose phosphates), compounds containing three carbon atoms (alanine and serine), and compounds (not including malic acid) containing four and five carbon atoms (fumaric acid, succinic acid, citric acid, glutamic acid, aspartic acid and threonine). Sucrose and malic acid have been plotted separately. In addition to these materials containing approximately 95% of the total radioactivity present on the paper, other compounds such as glycollic acid and glycine were observed in small quantity.

A break-up of the total organic phosphates was attempted. Three phosphates (phosphoglyceric acid and two different hexose phosphates) were well defined in the case of photosynthesis. The data for appearance of radioactivity in these phosphates showed that radioactivity was greatest in phosphoglyceric acid and decreased with time (the amount of radioactivity present in phosphoglyceric acid being almost equal to the sum of that present in the two hexose phosphates).

In summarizing the data on the organic phosphates, one point regarding the experimental method should be emphasized. In all experiments a sample taken at a definite time was centrifuged in the dark before alcohol extraction. The data of Benson and Calvin (6) show that radioactivity present in organic phosphates decreases markedly if the photosynthesis (at high light intensity) is followed by a dark period in carbon dioxide-free air. For example, one set of data shows that, after 30 seconds of photosynthesis (barley), 59% of the radioactivity is present in organic phosphates and 15% in sucrose. If the 30 second photosynthesis period is followed by a two minute dark period, 42% of the radioactivity is present in the organic phosphates and 34% in sucrose. Although the sum of the radioactivity in both components remains the same (about 75%) a dark period decreases phosphate activity with a corresponding increase in sucrose activity. Under the same conditions the percentage of radioactivity in e.g. malic acid is unchanged by the dark period. In the present experiments, the dark period (3-4 minutes) would be expected to decrease the amount of radioactivity present in the organic phosphate, especially at the early sampling times. Accordingly, a dotted line has been drawn (Figures 5, 6, 7) to extrapolate the phosphate curves and indicate the probable intercept on the vertical axis.

DISCUSSION OF RESULTS

Some conclusions may be drawn concerning the mechanism of carbon dioxide uptake. On theoretical and kinetic reasoning there must be at least one, and perhaps more, compounds whose appearance curves must start at some finite value and decrease with time. If carbon dioxide is fixed in a single compound and all other compounds are formed from it, there should be a single finite intercept at zero

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(6)   A. A. Benson and M. Calvin, J. Exper. Bot., (in press).

time and it should represent 100% of the total fixed carbon. If carbon dioxide is fixed in more than one compound by independent reactions, extrapolation will yield more than one finite intercept at zero time with the curve having a negative initial slope. The relative values of these intercepts will correspond to the relative rates of the carbon dioxide fixation reactions.

An examination of the present data shown in Figures 5, 6 and 7 shows that one compound which has a pronounced initial negative slope is malic acid. It also appears that a second group of compounds (the organic phosphates) when extrapolated as shown by the dotted line, has a negative slope. Among the organic phosphates, phosphoglyceric acid predominates.

The conclusion is thus reached that there are at least two independent reactions by which carbon dioxide is fixed. The first, and most pronounced under the present very low light intensity conditions, leads to malic acid. The second leads to phosphoglyceric acid and the other organic phosphates.

This conclusion is in contrast to the results observed at higher light intensities (5). Under these circumstances the compound first to appear radioactive is phosphoglyceric acid. This is then followed by appearance of radioactivity in malic acid. Thus, the reactions leading to phosphoglyceric acid are faster at higher light intensities. The reactions leading to malic acid are faster at very low light intensities.

From Figures 5, 6 and 7 it will be seen that not only do malic acid and the organic phosphates have an initial negative slope, but, in some cases, the three carbon atom compounds and the four plus five carbon atom compounds have a negative slope. The three carbon atom compounds are indirectly related to phosphoglyceric acid via pyruvic acid. The four and five carbon atom compounds are intermediates characteristic of the well-known Krebs respiratory (oxidation) cycle which involves malic acid.
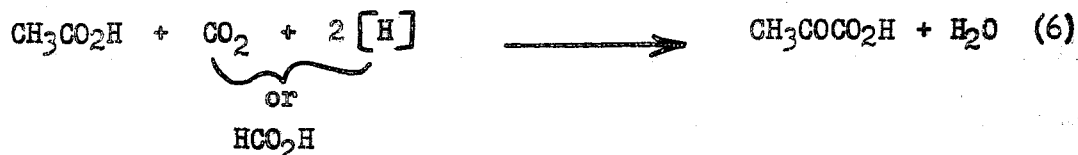
Carbon dioxide fixation reactions leading to the two main compounds (malic

acid and phosphoglyceric acid) are known (7). The first type, β-carboxylation, the Wood-Werkman reaction, involves addition of carbon dioxide <u>beta</u> to a carbonyl group. The enzyme catalyzing such a reaction

$$2[\text{H}] + CO_2 + \text{pyruvate} \rightleftharpoons \text{malate} \qquad (5)$$
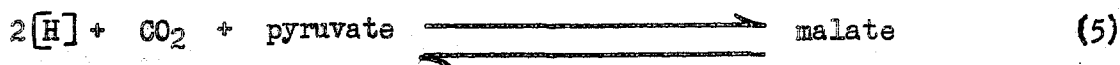
has been found to occur (8) in many plants. The second type of reaction is an α-carboxylation reaction and involves addition of carbon dioxide <u>alpha</u> to a carboxyl group. Several examples of this type are known.

The changes in the carbon skeleton are represented by:

$$CH_3CO_2H + \underbrace{CO_2}_{\text{or}} + 2[\text{H}] \longrightarrow CH_3COCO_2H + H_2O \qquad (6)$$
$$HCO_2H$$

and

$$CH_3CH=0 + CO_2 \longrightarrow CH_3COCO_2H \qquad (7)$$

the latter of which (equation 7) is simply the reverse of the carboxylase reaction. The actual reacting species and intermediate products of these reactions are almost certainly phosphate derivatives.

In order to account for the appearance of radioactivity in phosphoglyceric acid, a two-carbon atom compound which is to be carboxylated by an α-carboxylation type reaction is necessary. The two carbon acceptor from which phosphoglyceric acid

(7) See e. g. H. G. Wood, Physiol. Rev., <u>26</u>, 198 (1946).

(8) E. Conn, B. Vennesland and L. M. Kraemer, Arch. Biochem., <u>23</u>, 179 (1949).

is probably formed is not yet known. It has been pictured (10) as resulting from a closed cycle of reactions involving oxalacetic acid from which the malic acid is formed. An alternative source of the two carbon acceptor, which is not eliminated by these experiments, involves the direct reduction of carbon dioxide as a one carbon coumpound. This may be followed by the combination of this reduced one carbon compound with another reduced one carbon compound or with carbon dioxide to form the two carbon acceptor. This suggestion is subject to the very definite limitation that the steady state concentration of the reduced one carbon intermediate must be extremely small.

It may be added that, although all compounds formed under the four conditions studied are the same, they are not necessarily identical substances. Differences in radioactive carbon distribution may occur in all four cases. No information on this point was obtained in the present investigation.

SUMMARY

1. A comparison of the rates of fixation of Carbon 14 dioxide in algae for the processes of photosynthesis, photoreduction and the hydrogen-oxygen-carbon dioxide dark reaction has been made.

2. For the same series of experiments, rates of incorporation of tracer carbon into the separate soluble components using the radiogram method have been determined.

3. The mechanism of carbon dioxide uptake has been shown to occur via two distinct paths.

4. In all cases studied, essentially the same compounds appear radioactive. The distribution with time, however, differs markedly.
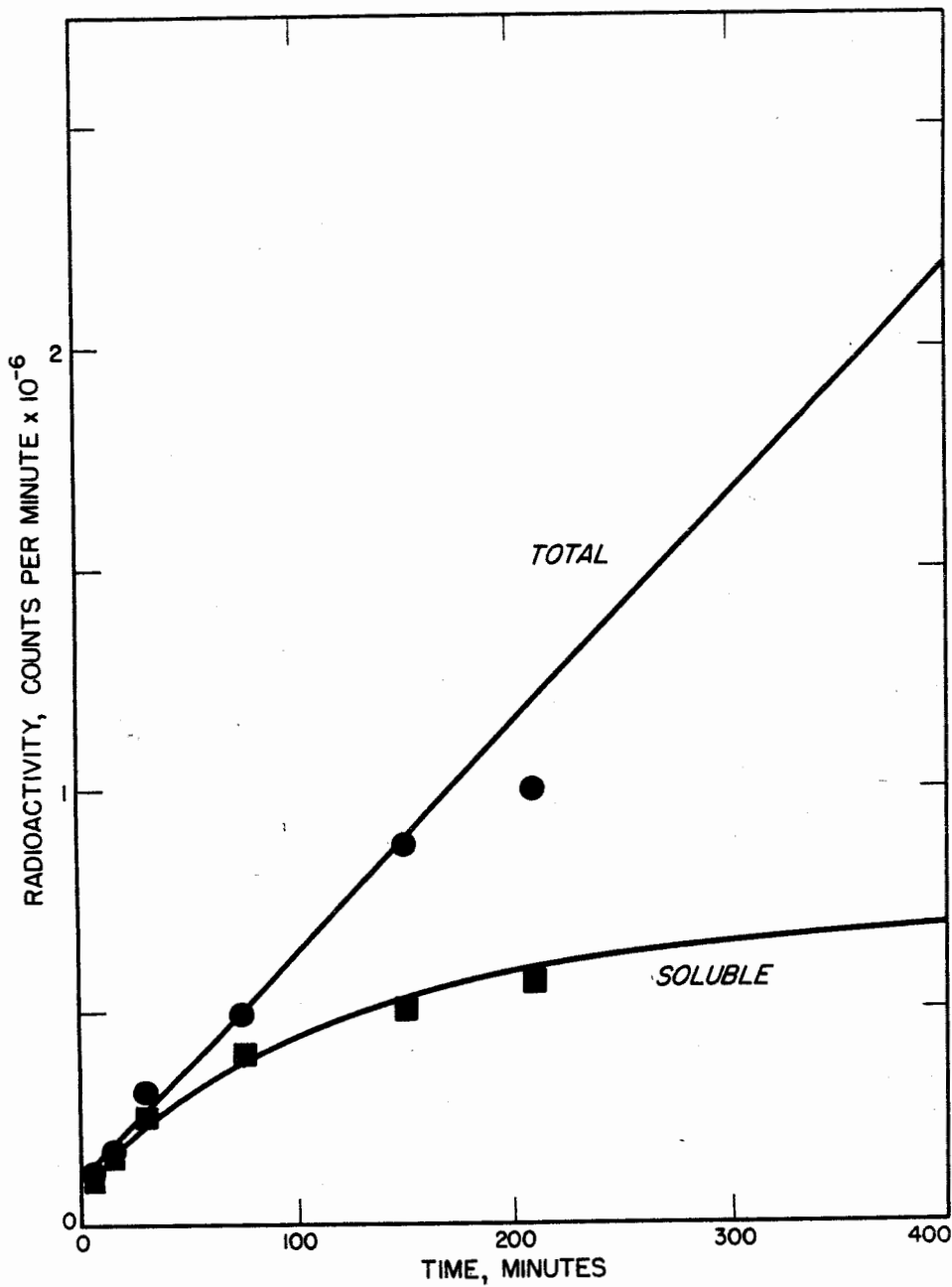
(10)　M. Calvin, J. Chem. Educ., _26_, 639 (1949)

Figure 1.

Photosynthesis. Rate of incorporation of tracer carbon. Temperature, 20.7°.
Scenedesmus D₃, 0.10 cc cells in 2.9 cc 0.05 M KH_2PO_4. Air atmosphere. Light
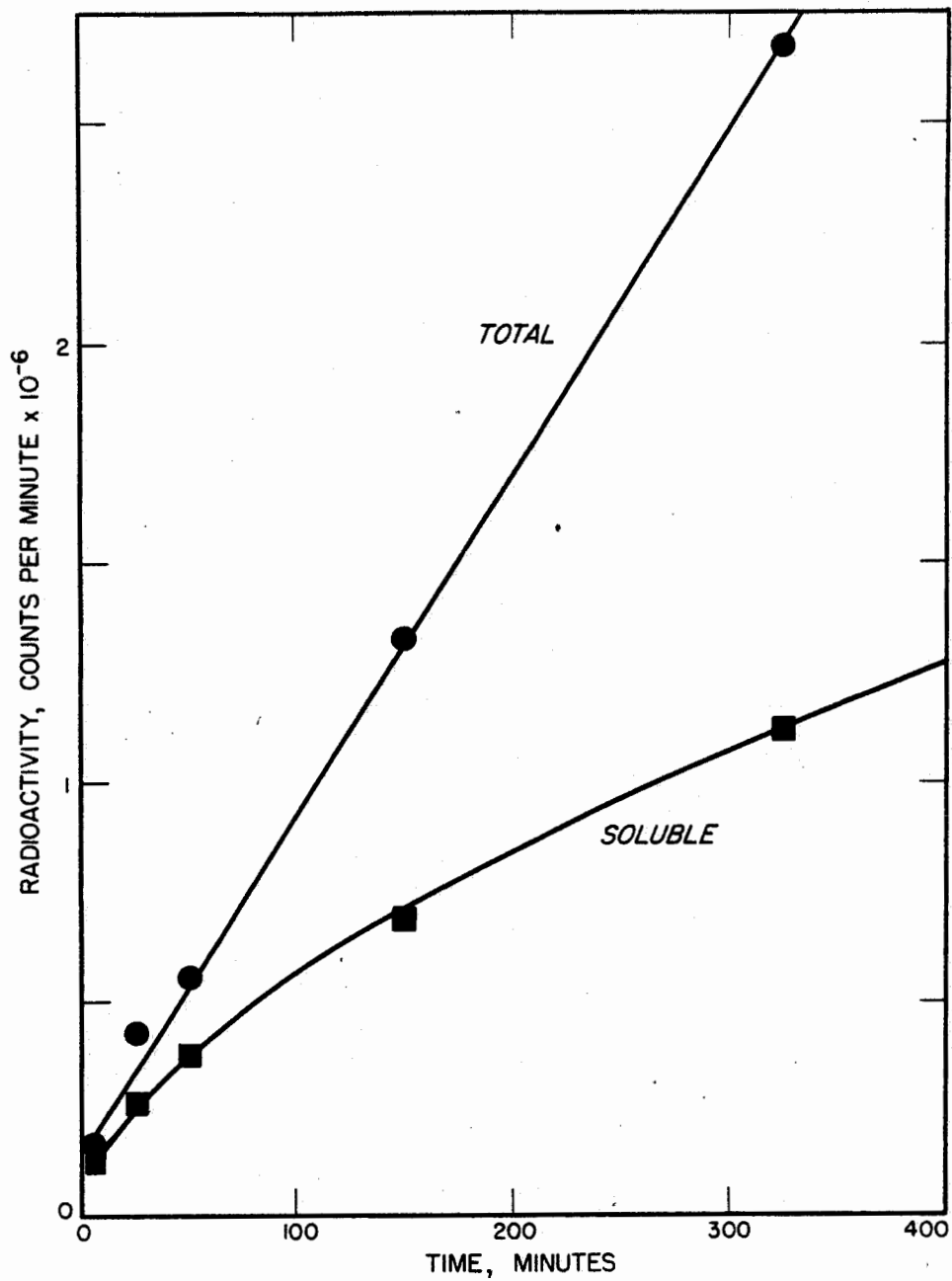intensity, 790 ergs/cm²/sec.

Figure 2.

Photoreduction. Rate of incorporation of tracer carbon. Temperature, 20.7°.
Scenedesmus D_3, 0.10 cc cells in 2.9 cc 0.05 M KH_2PO_4, adapted 16 hours in H_2
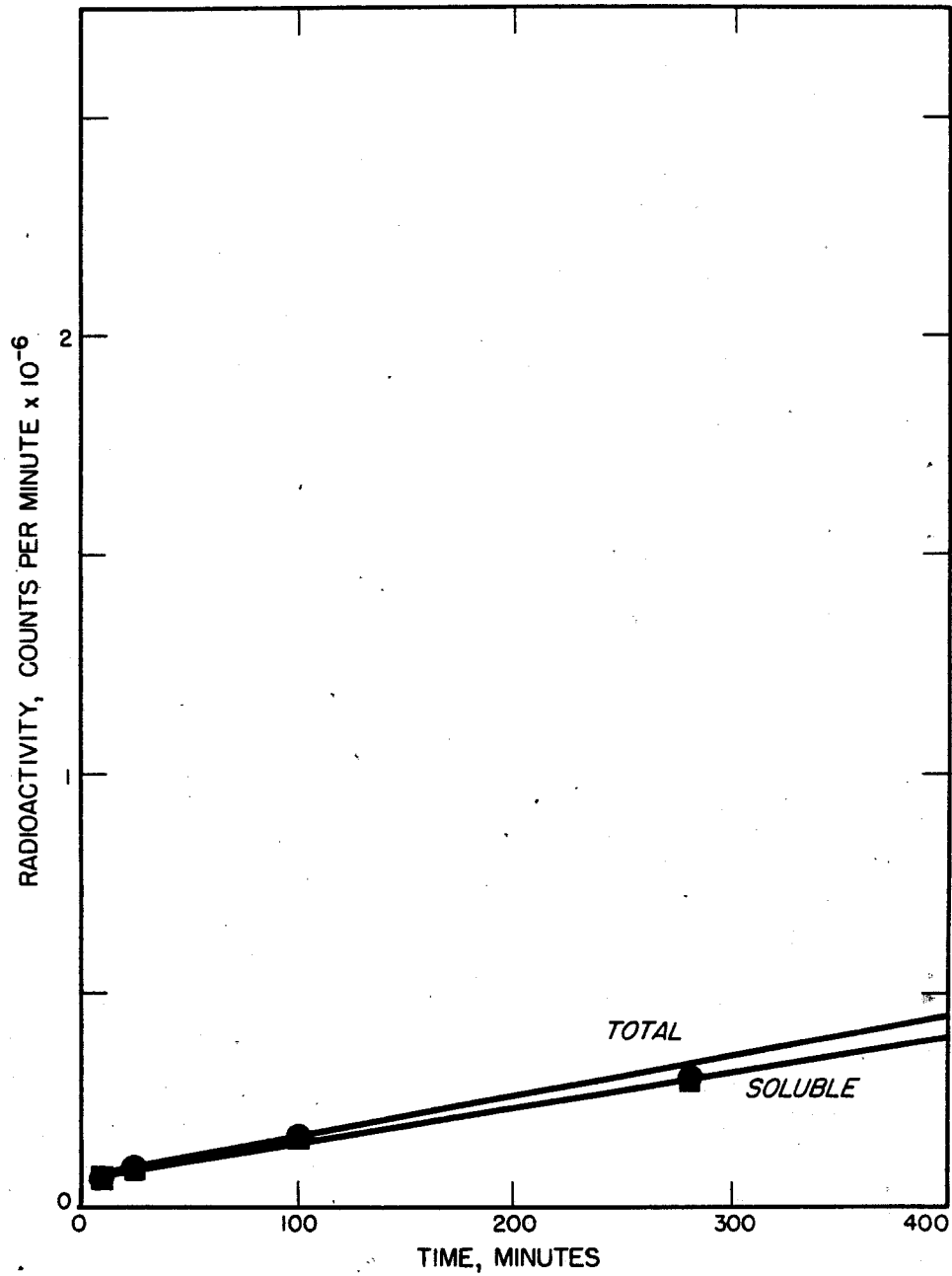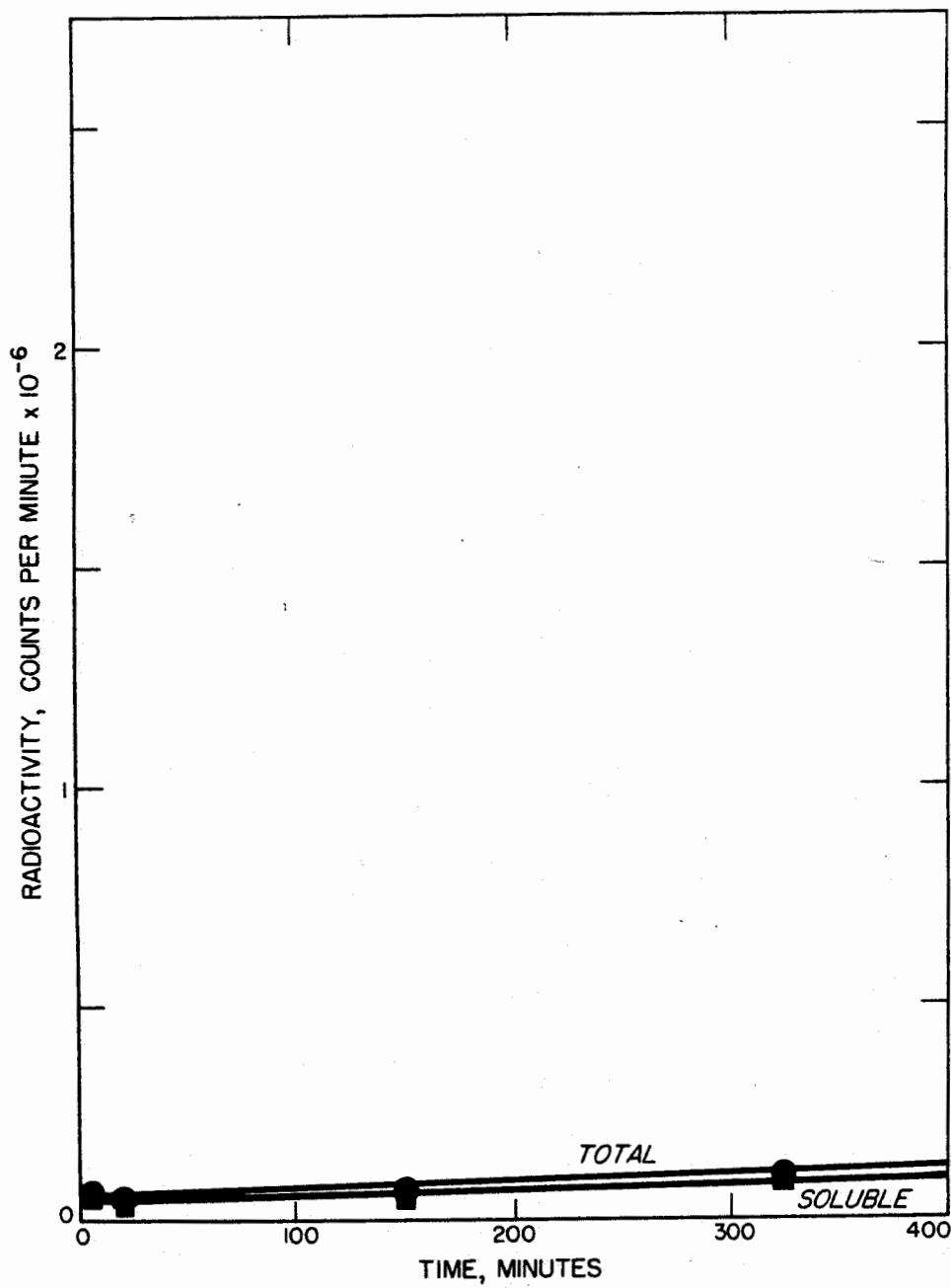atmosphere. Light intensity, 790 ergs/cm²/sec.

Figure 3.

Hydrogen-oxygen-carbon dioxide dark reaction. Rate of incorporation of tracer carbon. Temperature 20.7°. Scenedesmus D_3, 0.10 cc cells in 2.9 cc 0.05 M KH_2PO_4, adapted 16 hours in H_2 atmosphere.

Figuré 4.

Hydrogen-carbon dioxide dark reaction. Rate of incorporation of tracer carbon. Temperature, 20.7°. Scenedesmus D_3, 0.10 cc cells in 2.9 cc 0.05 M KH_2PO_4, adapted 16 hours in H_2 atmosphere.

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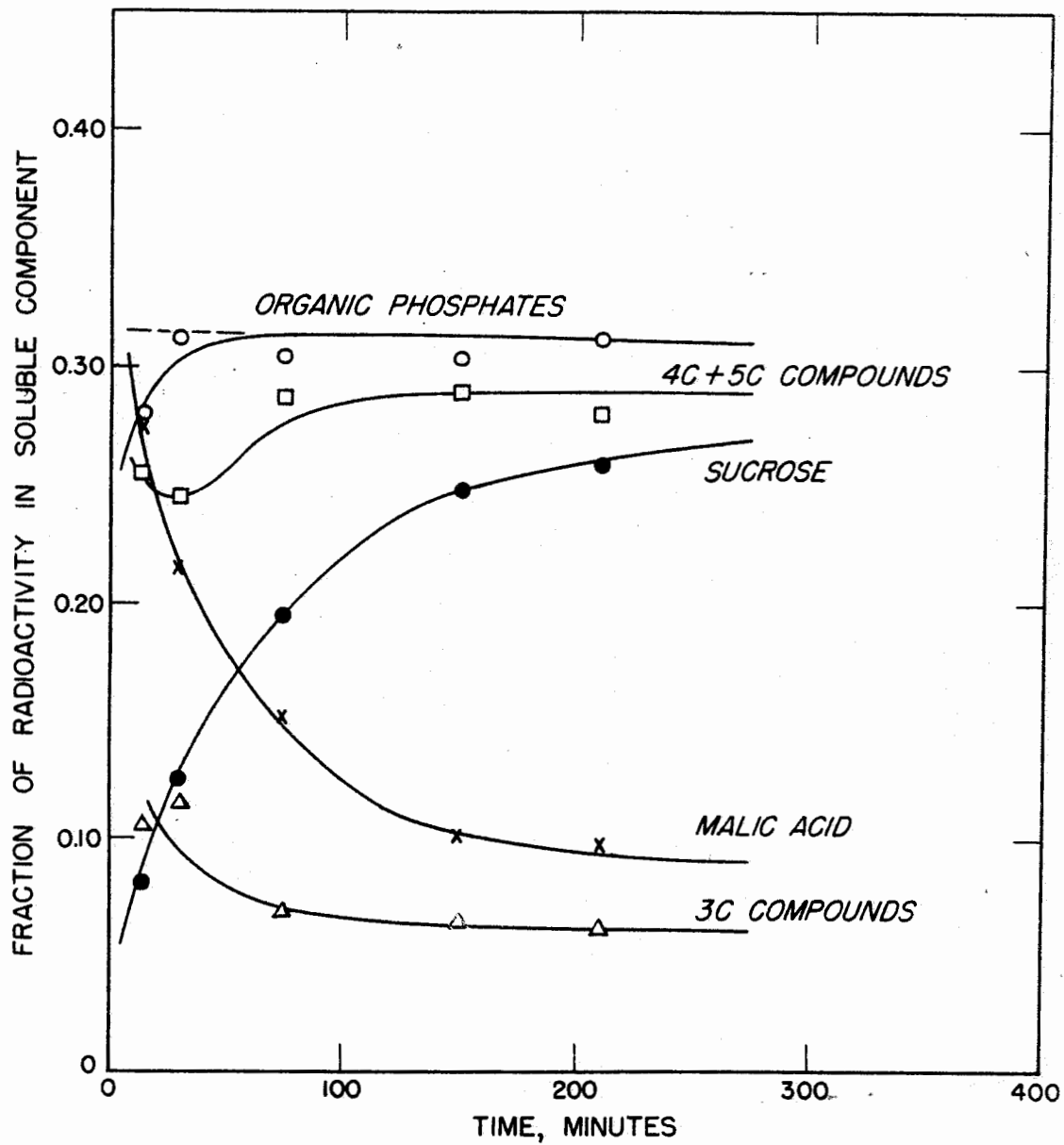
Figure 5.

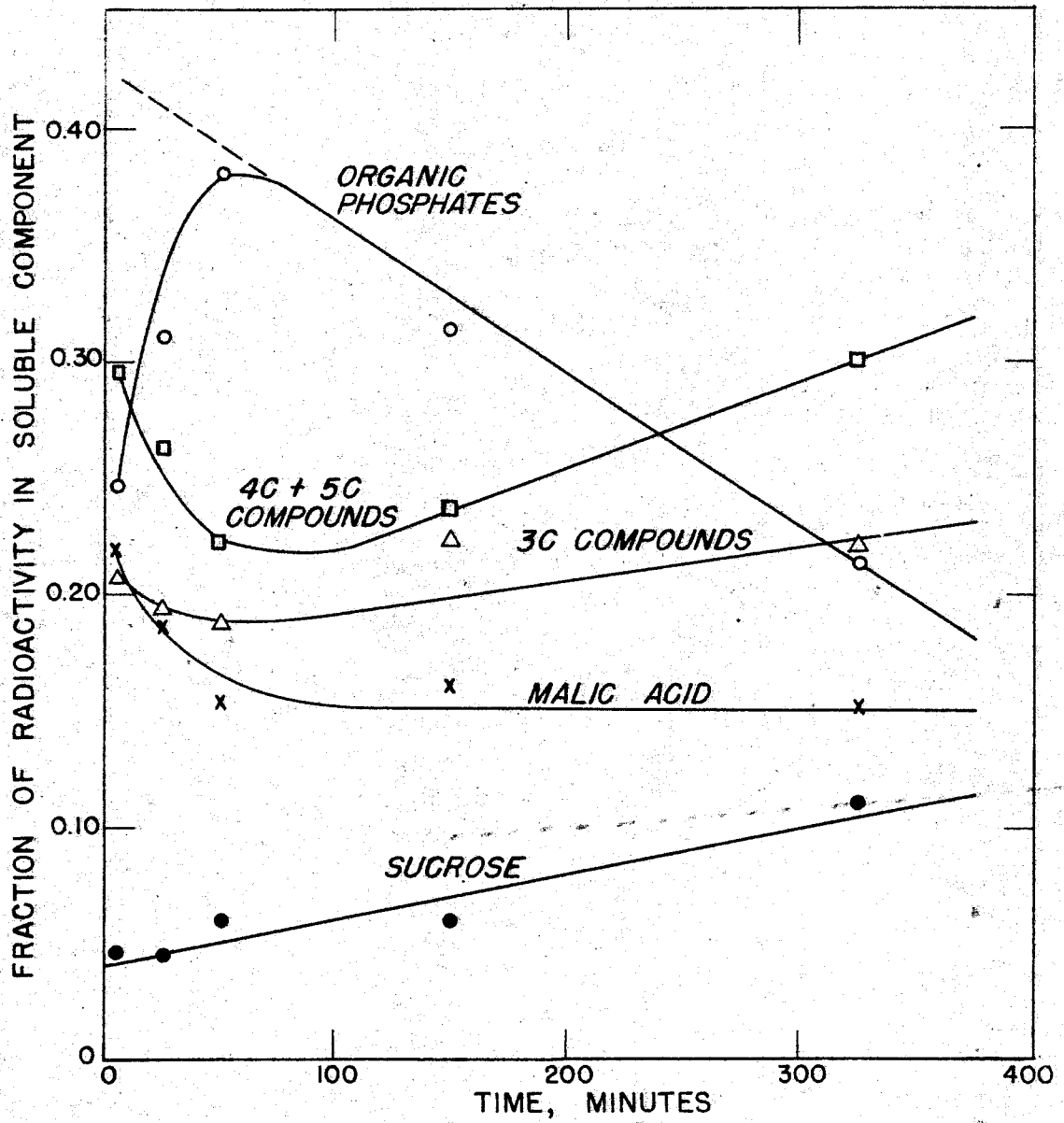Photosynthesis. Rate of distribution of tracer carbon in components of soluble fraction.

Figure 6.

Photoreduction. Rate of distribution of tracer carbon in components of soluble fraction.
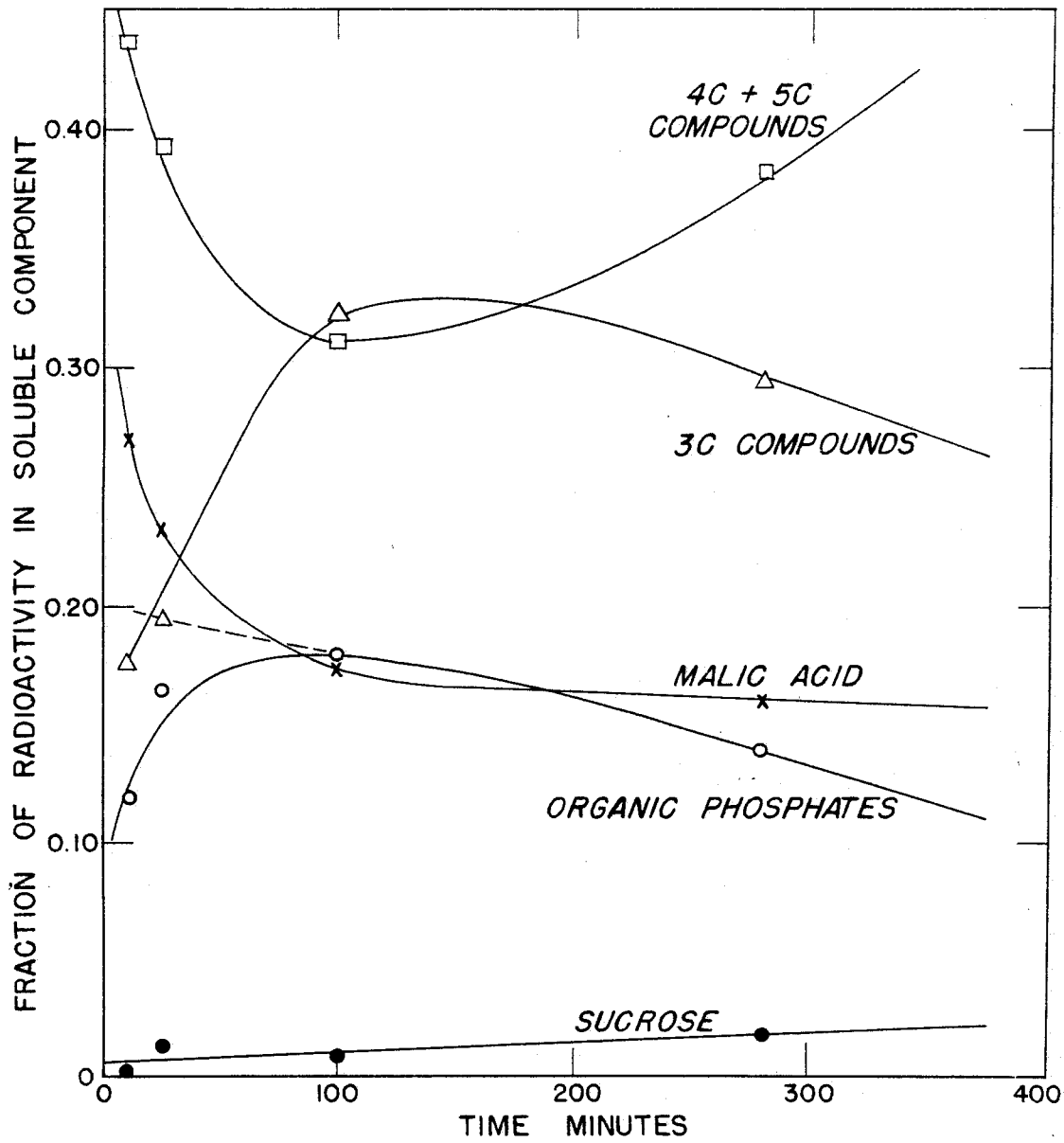
Figure 7.

Hydrogen-oxygen-carbon dioxide dark reaction. Rate of distribution of tracer carbon in components of soluble fraction.
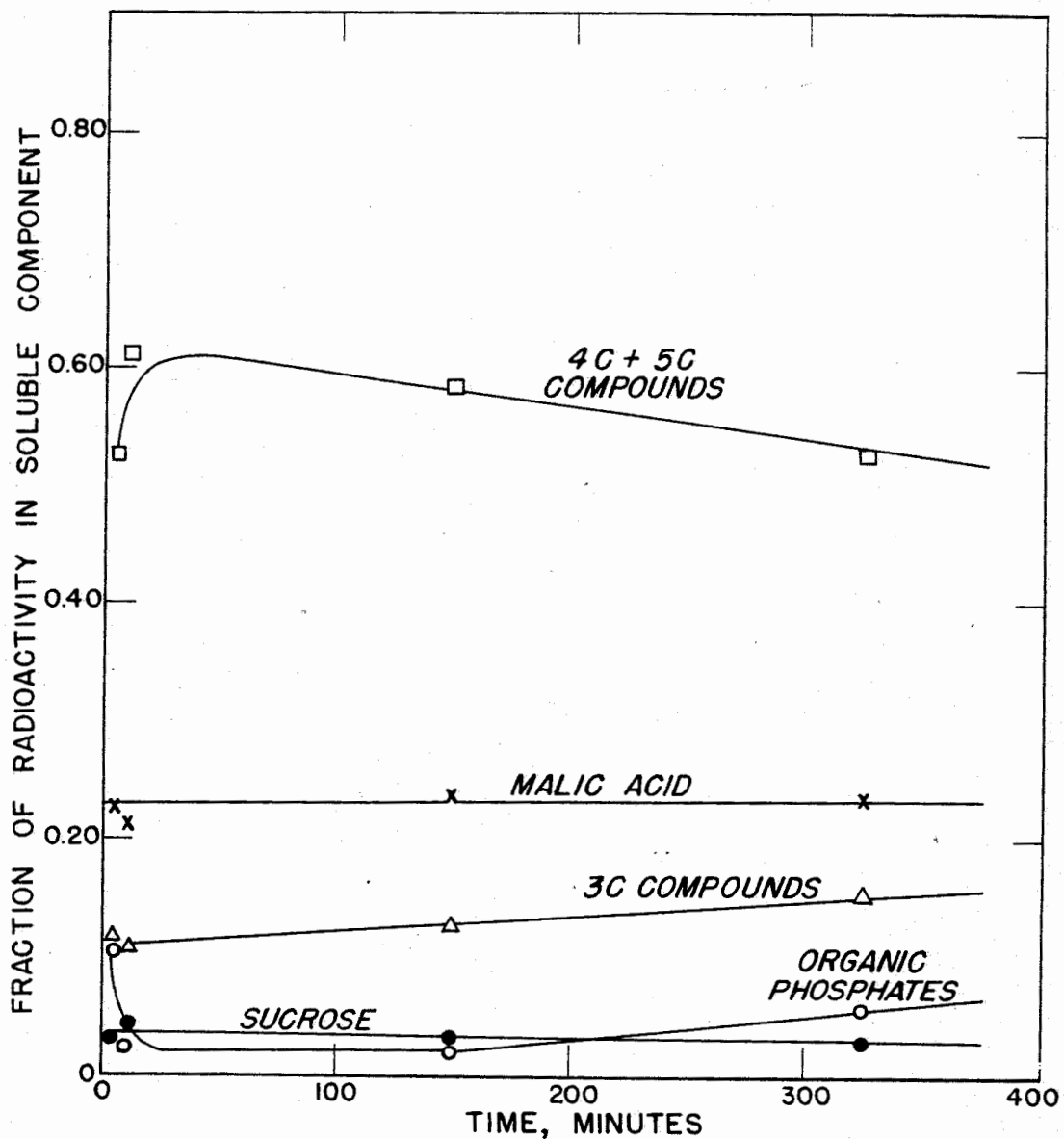
Figure 8.

Hydrogen-carbon dioxide dark reaction. Rate of distribution of tracer carbon in components of soluble fraction.