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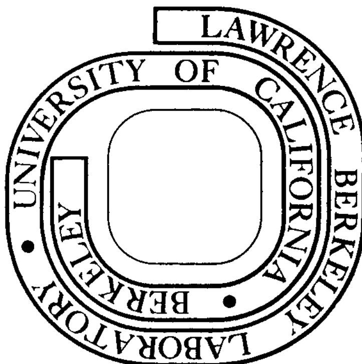


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Running Title: STARCH METABOLISM IN CHLOROPLASTS

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# LIGHT-DARK REGULATION OF STARCH METABOLISM IN CHLOROPLASTS

# II. EFFECT OF CHLOROPLASTIC METABOLITE LEVELS ON THE FORMATION OF ADP-GLUCOSE BY CHLOROPLAST EXTRACTS[1]

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ABBREVIATIONS:

| | |
|---|---|
| PGA | 3-phosphoglycerate |
| G6P | glucose-6-phosphate |

[1]This work was supported in part by the Biomedical and Environmental Research Division of the U. S. Department of Energy

[2]Recipient of a post-doctoral fellowship of the Deutsche Forschungsgemeinschaft (DFG)

ABSTRACT

The soluble fraction of lysed chloroplasts synthesizes ADPglucose from added $^{14}C$-glucose-6-phosphate and ATP. The rate of ADPglucose formation is determined as a function of the levels of metabolites and ions found in whole chloroplasts. During light-dark transition, ADP-glucose formation is governed by a fast drop in ATP (1.0 mM in the light, 0.2 mM in the dark) and by the change in stromal pH (pH 8.0 in the light, 7.3 in the dark). A slower but more complete inhibition of ADPglucose formation is caused by the decrease in 3-phosphoglycerate (3.6 mM in the light, 1.4 mM in the dark), with Pi being constant at 4 mM. An observed increase in the hexose monophosphate concentration, which occurs at the same time, stimulates the rate of ADPglucose formation, but to only a very low level. Starch formation from ADPglucose can be observed only if either chloroplast pellet (containing starch) or boiled starch granules are added as primer. The formation of UDPglucose, often found associated with chloroplast preparations, is shown to be due to contamination by cytoplasmic enzymes.

INTRODUCTION

In the preceding paper we measured changes in metabolite levels in isolated intact chloroplasts in order to determine whether light-dark regulation os starch synthesis in such chloroplasts does in fact occur via the well-known modulation of ADPglucose formation by glycolytic intermediates and Pi (15). We found that after several minutes in the light, the level of Pi, which is a potent inhibitor of ADPglucose pyrophosphorylase (9,10,21,23), stayed constant throughout a following light-dark-light cycle. The level of PGA, which stimulates ADPglucose formation (9, 10,21,23), did decrease dramatically in the dark, but the concentration of hexose monophosphates, which also stimulate ADPglucose pyrophosphorylase (9,10,21,23), increased in the dark by a slightly larger factor (15). Furthermore, the concentrations of these metabolites changed only slowly when compared with the fast light-dark kinetics of ADPglucose levels in whole cells (2) or of starch formation in whole chloroplasts (15).

These observations raise some questions with respect to the role of these metabolites in the fast light-dark regulation of starch formation. Therefore we have now investigated the formation of ADPglucose from $^{14}C$-GGP and ATP in the soluble fraction of lysed chloroplasts at the different metabolite levels which we found in whole chloroplasts in the light and dark. In addition, we studied the effects of several other factors which are known to regulate Calvin cycle enzymes (16), such as different levels of ATP, NADPH, $Mg^{++}$ and pH under conditions which are now known to exist in whole chloroplasts, as well as the effects of thiol compounds known to simulate some regulatory effects of analogous physiological thiol compounds in chloroplasts (16).

Requirements for the formation of starch by the chloroplast extract were examined. Also, the formation of UDPglucose was measured in the extract and compared with its formation in washed and unwashed intact chloroplasts.

## MATERIALS AND METHODS

Chemicals and Enzymes. U-$^{14}$C-glucose was obtained from New England Nuclear (NEN) and lyophilized hexokinase from Calbiochem (stock #376 811). Phosphoglucomutase (E.C. 2.7.5.1.) from Calbiochem (#52508) was dialyzed for 5 h at 0°C against 250 ml of lysing solution (see below), containing in addition 1 mM DTT, and used within 2 h.

Plant Material. Spinach was grown in vermiculite fertilized with Hoaglands solution, under artificial light (3000 ft-c) with an 8 h light period and a 16 h dark period at a temperature of 13°C. Chloroplasts were isolated from young leaves according to the method of Jensen and Bassham (13). Chlorophyll was measured following the procedure of Arnon (1).

Chloroplast Extract. For preparing a chloroplast extract, a suspension of freshly isolated whole chloroplasts in a volume of 2 ml and containing 2 mg chlorophyll was centrifuged for 1 min at 1000 g (4°C) and the pellet was resuspended in 1.2 ml of a lysing solution containing HEPES-NaOH pH 7.6, 1 mM $MgCl_2$ and 1 mM EDTA. After 10 min at 0°C, the suspension was centrifuged for 15 min at 27,000 g (4°C). The resulting straw-colored supernatant contained the soluble components from the chloroplasts and had an average protein content of 5 mg protein in 1.2 ml. It was stored on ice until it was used for the experiments (usually within 1 h after isolation). The pellet was resuspended in 1.2 ml lysing solution and used for chlorophyll determination and for experiments, as indicated in the legends of the figures.

Preparation of U-labeled $^{14}C$-glucose-6-phosphate. $^{14}C$-glucose-6-phosphate was prepared from U-$^{14}C$-glucose. 2 mg of hexokinase containing 10 I.U. and stochiometric amounts of ATP were added to 0.5 ml of $^{14}C$-glucose (1.25 mCi/4.1 μmol) in the lysing solution described above. After 1 h at room temperature, the solution was heated for 1 min in boiling water. The protein precipitate was spun down and separated from the supernatant. The conversion of glucose into G6P was checked by two-dimensional paper chromatography (see below). Usually, more than 98% of the added $^{14}C$-glucose was converted into G6P. After that, unlabeled G6P was added to give a specific activity of 50 μCi/μmol, and the solution was stored at -15°C until needed.

Incubation Conditions. 300 μl of the chloroplast extract was added to the lysing solution containing 2 to 6 mM $^{14}C$-G6P and different metabolites in a final volume of 500 μl. The solution was assayed in serum-stoppered flasks in a water bath at 21°C. At timed intervals 50 μl aliquot samples were removed with microsyringes and injected into 450 μl methanol (final methanol concentration 90%).

Analysis of Products. Products of the metabolism of $^{14}C$-G6P were separated by descending paper chromatography and radioautography (13). 150 μl samples of the protein-methanol mixture were spotted on Whatmann #1 paper and the chromatograms were developed in two dimensions as described earlier (20).

## RESULTS AND DISCUSSION

Sites of the Formation of UDPglucose and of ADPglucose. In experiments with chloroplast extracts and added $^{14}C$-G6P and ATP we observed not only ADPglucose formation, as expected, but also UDPglucose formation. ADPglucose is the glucosyl-donor for starch formation (6,18,22), whereas UDP-glucose is mainly used for the formation of sucrose or sucrose-phosphate (5,11).

Although it is known that sucrose cannot penetrate the chloroplast envelope and that it therefore has to be synthesized in the cytoplasm in order to function as a transport metabolite (12), there still exist some conflicting results on the site of sucrose synthesis (3,4,7).

In our experiments, the observed UDPglucose formation could be due either to chloroplastic enzymes or to a contamination of our chloroplast extract with cytoplasmic UDPglucose pyrophosphorylase. It is known that hexose monophosphates cannot penetrate the chloroplast envelope (12). It was therefore of interest to investigate possible formation of UDPglucose from added $^{14}$C-G6P and ATP in a preparation of highly intact chloroplasts. In this case, it should be expected that any UDPglucose formation would be due to contamination with several cytoplasmic enzymes, and it should be reduced by simply washing the chloroplasts. On the other hand, if UDPglucose could be synthesized by chloroplastic enzymes, its formation should be greatly enhanced by lysing the intact chloroplasts.

All experiments were carried out in the dark, and only G6P, ATP or UTP and phosphoglucomutase were added. Washing the intact chloroplasts reduced the rate of UDPglucose formation dramatically (Fig. 1) whereas formation of ADPglucose was not affected. After lysis of unwashed, intact chloroplasts, the rate of UDPglucose formation remained unaffected, but ADPglucose formation was strongly increased. This experiment demonstrates clearly that UDPglucose was formed by cytoplasmic enzymes contaminating our chloroplast preparation, whereas ADPglucose formation occured within the chloroplasts.

<u>Effect of chloroplastic levels of PGA, Pi and Hexose Monophosphates on ADPglucose formation</u>. In our preceding work we found that the level of PGA in illuminated intact chloroplasts was about 3.6 - 4 mM (15). At this concentration of PGA, and in the absence of Pi, the rate of ADPglucose

formation by chloroplast extracts was high, reaching values between 10-50 μg atoms carbon/mg chloroplyll x h (compare Table I, a and Fig. 2). These rates were as high or even higher than the rates of starch formation in whole chloroplasts (15).

If Pi is present at the concentration found in intact chloroplasts in the light and dark (3.6 mM), the rate of ADPglucose formation was lower, reaching values between 1.5 to 6 μg-atoms carbon/mg chlorophyll x h (compare Table I, b and Table II,c,d). But even these lower rates are still in the range of the rates of starch formation in whole chloroplasts, which, under our conditions varied between 4-10 μg atoms carbon/mg chlorophyll x h. If Pi was kept constant at 3.6 mM (as it is in the whole chloroplast), and the concentration of PGA was reduced to the dark level of about 1.4 mM, the rate of ADPglucose formation decreased dramatically by about 90% (Table I, c). These experiments were carried out at a hexose monophosphate concentration of 2 mM. However, we knew that the level of hexose monophosphates increases in the dark in isolated chloroplasts from 2 to 6 mM (15). This increase might be expected to balance the inactivation of ADPglucose pyrophosphorylase caused by the decreased level of PGA. However (Table I), although changing the hexose monophosphate concentration from 2 to 6 mM increased the rate of ADPglucose formation three-fold, the resulting rate was only about 12% of the rate in the presence of 3.6 mM PGA (with other factors constant).

These results demonstrate that a change in the level of PGA as it occurs in intact chloroplasts during light-dark-light transition (15), leads to a fairly effective regulation of ADP-glucose pyrophosphorylase, despite the fact that [Pi] remains constantly high and the hexose monophosphate level undergoes changes in the opposite direction. Nevertheless, the changes in the level of PGA during light-dark-light transition were found to be much

slower than the changes in rate of starch formation (15) or, even more significant, the changes in the rate of ADPglucose formation in vivo (2). Other factors governing the fast light-dark-light change of the rate of ADPglucose formation therefore must be considered.

Further Factors Affecting the Rate of ADP-glucose Formation. It is known that the level of ATP in whole chloroplasts decreases from about 1 mM in the light to 0.2 mM in the dark within 8-15 sec, and in the light it increases as rapidly (14,24,26). Further, the extensive work of Preiss and coworkers (9,10,21,22) has shown that the activity of ADPglucose pyrophosphorylase increases with higher levels of ATP, depending on the activation state (induced by PGA and Pi). It was therefore of interest to determine how the rates of ADP-glucose formation would respond to the changes of ATP under the conditions which were found to exist in whole chloroplasts during light-dark-light transition. With chloroplastic enzymes, the rate of ADPglucose formation showed a nearly linear response to changes in ATP concentration when the concentrations of PGA, Pi, and hexose monophosphates were those found to exist in the whole chloroplast in the light (15, Table II, a). This indicates that the effect of ATP concentration is by mass action and not due to allosteric regulation under these conditions.

Among other factors which are known to regulate the activity of several Calvin cycle enzymes during light-dark transition are changes in stromal pH in the range of pH 7.3-8.0 (25), changes in the $Mg^{++}$ concentration, activation by reduced thiol groups or by the concentrations of NADPH and $NADP^+$ (16,17). The role of all these factors for a possible light-dark regulation of ADP-glucose formation was studied and the results are listed in Table II, b-d. It is clear that, besides the ATP level, only the pH of of the medium has a significant effect on the rate of ADPglucose formation.

In fact, a change in the pH from 8.0 to 7.3 as it occurs in whole chloroplasts from light to dark (25), reduced the rate of ADP-glucose formation to less than half. All other factors had no significant effect.

Light-dark Regulation of ADPglucose Formation in Chloroplasts. During the light-dark transition, the rate of ADPglucose formation in chloroplasts seems to be governed principally by three factors: i) a fast drop in [ATP] from 1 mM in the light to 0.2 mM in the dark, which by itself could cause an 80% reduction in the rate of ADPglucose formation, ii) a rapid change in the stromal pH from 8.0 in the light to 7.3 in the dark, which alone reduces ADPglucose formation to 40% of the light value. (Since the ATP formation is directly coupled to the proton gradient between stroma and thylakoids, the change in stromal pH should be expected to be at least as fast as the change in [ATP])., iii) A slower decrease in the concentration of PGA from about 3.6 mM in the light to 1.4 mM in the dark (at a constant level of Pi) which inhibits ADPglucose formation to less than 10% of the light value. The combined effect of these three factors would reduce the rate of ADPglucose formation enough to affect regulation of starch formation during the light-dark transition, despite the fact that the hexose monophosphates undergo a change in concentration which is opposite to the change in [PGA].

Starch Formation by Chloroplast Extracts. Despite the high rates of ADPglucose formation in our experiments with chloroplast extracts, there was usually no starch formation at all. This is not surprising, because starch formation from ADPglucose requires the presence of both a primer (i.e. an $\alpha$-1,4-polyglucan) and starch synthetase. This enzyme exists

either tightly attached to starch granules (6) or in a soluble form (8). In our experiments, the chloroplast starch granules usually spin down with the chloroplast pellet. If part of that pellet was resuspended and added to a chloroplast extract with fully activated ADP-glucose pyrophosphorylase (i.e. at high [PGA] and without Pi), starch was formed from $^{14}$C-G6P and ATP at a rate of 2-3 μg atoms carbon/mg chlorophyll x h (Fig. 2). If boiled starch was added instead, the rate of starch formation was even higher, reaching values of more than 7 μg-atoms carbon/mg chlorophyll x h. It should be mentioned that in this experiment, chloroplast pellet or starch granules were added 12 min after the formation of ADPglucose had been started, in order to allow a sufficiently high pool of ADPglucose to be built up. In fact, as shown in Fig. 2 (left), the rate of ADPglucose formation was already decreasing after that time presumably due to a lack of ATP, and addition of starch or pellet reduced the net accumulation of ADPglucose even more (due to consumption of ADPglucose).

These experiments demonstrate that our chloroplast extract contains a high amount of soluble starch synthetase and that the rate of starch formation in this system is limited by the availability (and concentration) of primer. Such a lack of primer might well be a physiological situation in plants, e.g. after long periods of low light intensities or darkness. One might therefore speculate that the main purpose of an amylolytic starch breakdown in chloroplasts (19) would be to maintain a certain level of primer inside the chloroplasts, without keeping too much carbon in the form of immobile starch. This would guarantee a fast onset of starch formation even after longer periods of darkness.

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| Metabolites Added (mM) | ADPglucose Formed (μg-atoms/mg chlorophyll x h) |
|---|---|
| a) PGA 3.6; no Pi; G6P 2 | 13.74 |
| b) PGA 3.6; Pi 3.6; G6P 2 (= light conditions) | 1.47 |
| c) PGA 1.4; Pi 3.6; G6P 2 | 0.06 |
| d) PGA 1.4; Pi 3.6; G6P 6 (= dark conditions) | 0.18 |

Table I: Formation of ADPglucose by a chloroplast extract at the levels of Pi, PGA and hexose monophosphate (added as G6P) found in intact chloroplasts in the light or dark (15).

300 μl of chloroplast extract were added to a medium containing 0.025 M HEPES-NaOH pH 7.6; 1 mM $MgCl_2$; 1 mM EDTA; 1 mM ATP; U-$^{14}$C-glucose-6-phosphate and other metabolites as indicated in the table. The expressions "light-condition" or "dark-condition" are used with respect to the metabolite levels found in intact chloroplasts in the light or dark, respectively. All rates were calculated from the linear time course of ADP-glucose formation during 10 min and are based on the chlorophyll content of the amount of chloroplasts, from which the extract was obtained. For further details see Materials and Methods.

| Conditions | | ADPglucose formed (μg-atoms/mg Chlorophyll) |
|---|---|---|
| a) | ATP 1.0 mM | 1.34 |
| | ATP 0.2 mM | 0.18 |
| b) | $MgCl_2$ 12 mM | 0.95 |
| | $MgCl_2$ 1 mM | 1.34 |
| c) | pH 8.0 | 5.75 |
| | pH 7.3 | 2.25 |
| d) | +DTT 10 mM | 5.75 |
| | -DTT | 5.25 |
| e) | NADPH 1 mM | 1.80 |
| | $NADP^+$ 1 mM | 1.50 |

Table II. Formation of ADPglucose by a chloroplast extract, as influenced by different factors known to change in the chloroplast during light-dark transition (see ref. 16).

If not otherwise indicated, the reaction medium contained in a final volume of 500 μl: 300 μl chloroplast, 0.025 M HEPES-NaOH pH 7.6; 1 mM $MgCl_2$; 1 mM EDTA: 1 mM ATP, 2 mM U-$^{14}$C-glucose-6-phosphate; 3.6 mM PGA, 3.6 mM Pi and other compounds as indicated. DTT was used in section d), in order to simulate light activation of enzymes by thiol-groups (16). Each section (a-e) represents a separate experiment. Therefore the rates can be directly compared only within each section. For further details see Table I and Materials and Methods.

LEGENDS TO THE FIGURES

Fig. 1: Formation of UDPglucose (UDPG) and ADPglucose (ADPG) from U-$^{14}$C-glucose-6-phosphate and UTP and ATP, respectively, by normally isolated, intact chloroplasts ("intact"), by chloroplasts washed once ("intact, washed") and by lysed chloroplasts ("lysed"). For preparing the "washed" and "lysed" chloroplasts, a suspension of normally isolated chloroplasts was divided into two parts and centrifuged for 1 min at 1000 g (4°C). The supernatant was discarded and the pellet was suspended in a medium with sorbitol ("washed") or without sorbitol ("lysed"). In addition, this solution contained 0.025 M HEPES-NaOH pH 7.6, 1 mM $MgCl_2$, 1 mM EDTA. After all three suspensions were adjusted to the same chlorophyll content (250 µg chlorophyll), 300 µl of each preparation was added to the reaction medium, which contained in addition: 1 mM U-$^{14}$C-glucose-6-phosphate; 10 I.U. phosphoglucomutase; and 1 mM ATP or UTP. In addition sorbitol (0.33 M) was present only in the reaction medium for the intact (washed and normal) chloroplasts. The final volume of the reaction medium was 500 µl. The experiment was carried out in the dark to avoid interference witn photosynthesis. Since no effector (PGA) for ADPglucose pyrophosphorylase is added, the rate of ADPglucose formation even in the lysed chloroplasts is quite low.

Fig. 2: Formation of ADPglucose (ADPG) and starch from U-$^{14}$C-glucose-6-phosphate and ATP by chloroplast extract. The reaction mixture contained 300 µl of chloroplast extract, 0.025 M HEPES-NaOH pH 7.6; 5 mM $MgCl_2$, 1 mM EDTA; 1 mM ATP; 2 mM U-$^{14}$C-glucose-6-phosphate

and 3.6 mM PGA in a final volume of 500 μl. Where indicated, 30 1 of chloroplast pellet or 30 μl boiled starch (1.5 mg) was added. The chloroplast pellet was obtained by resuspending the sediment of the high-speed centrifugation step (see Materials and Methods) in 0.3 ml of the lysing solution. The pellet added to the reaction medium contained 275 μg chlorophyll and was equivalent to the amount of soluble chloroplast material in the reaction flask.

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Fig. 1

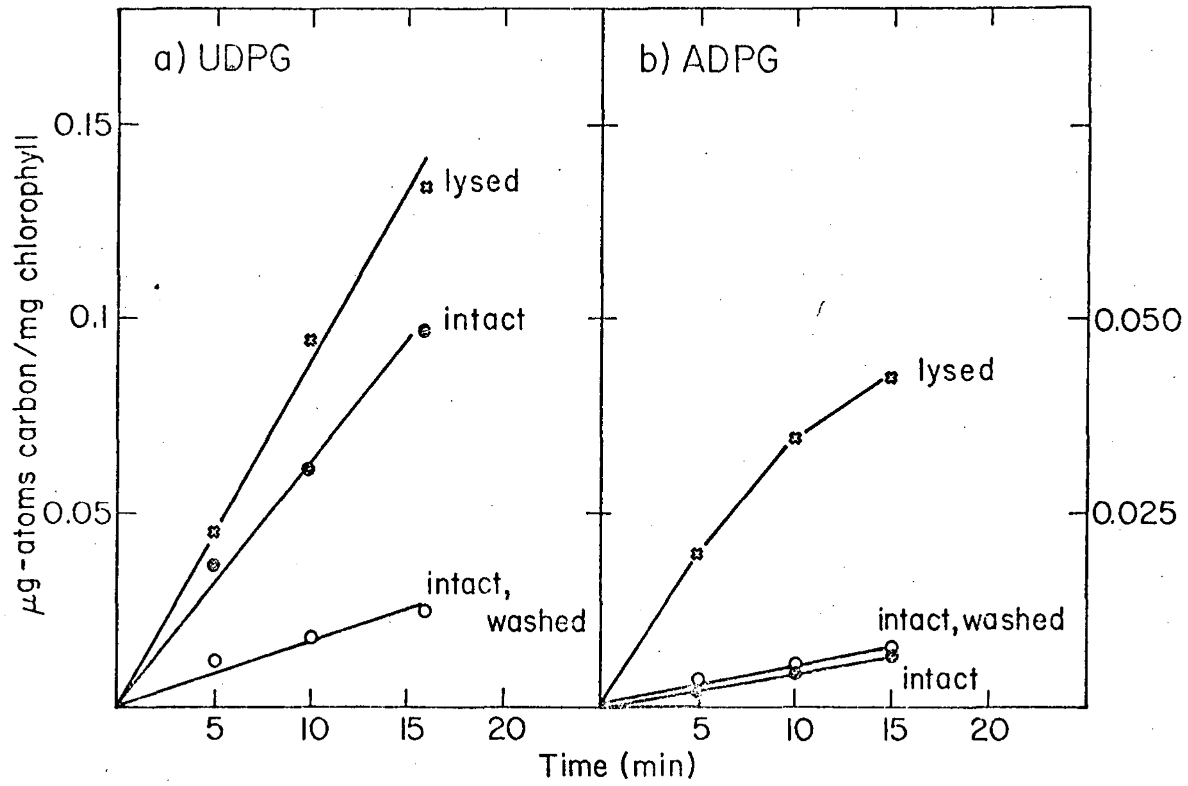
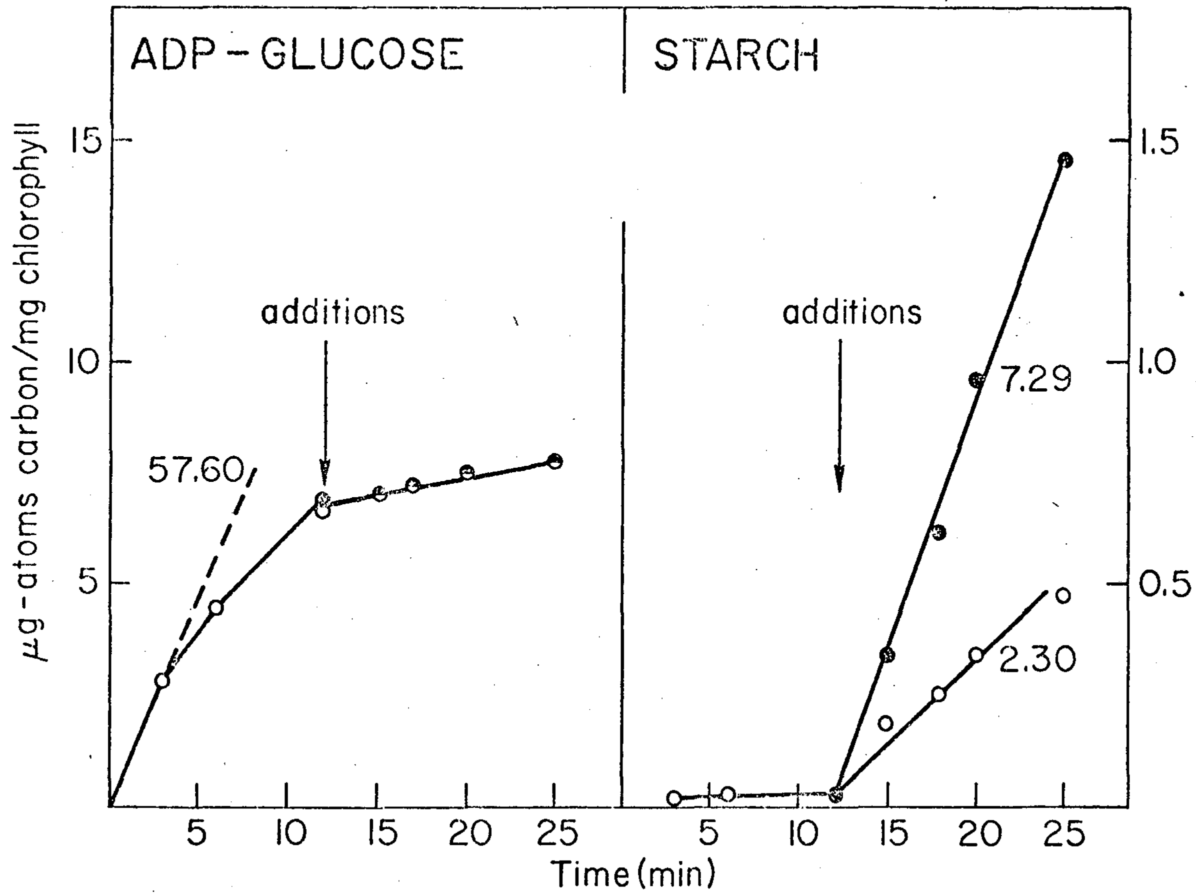


Fig. 2

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