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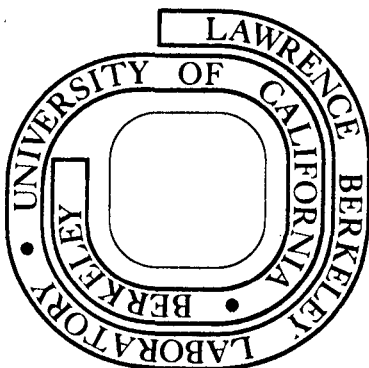
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Kinetics of Glucose Incorporation by Aphanocapsa 6714

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Photoautotrophic metabolism of CO₂ was compared with glucose metabolism in the facultative unicellular blue-green alga, Aphanocapsa 6714. Glucose-fed cells incorporated more ¹⁴C into phosphorylated sugar intermediates of the reductive and oxidative pentose phosphate cycles than autotrophic cells. The relative increases were: 140-fold in dark cells; 32-fold in dichlorophenylmethylurea (DCMU) inhibited cells; and 16-fold in cells assimilating glucose during photosynthetic carbon reduction.

On the other hand, incorporation of ¹⁴C from glucose into 3-phosphoglycerate and the amino acid pools of glutamate and aspartate was reduced in dark cells. Rates of protein synthesis in dark and DCMU inhibited cells was reduced 50 and 80% compared to photoautotrophic cells. In cells assimilating glucose during photosynthesis, rates of ¹⁴C incorporation into the two amino acids and protein was the same as in photoautotrophic cells.

Chase experiments, using an excess of ¹²C-glucose and CO₂ revealed slow turnover of carbon in dark cells, and intermediate turnover rates in DCMU inhibited cells, when compared to cells assimilating glucose during photosynthesis.

INTRODUCTION

An extensive study of unicellular blue-green algae (22) suggested that obligate photoautotrophy was the rule for this subgroup. However, recent studies have shown a few exceptions to this generalization.

Agmenellum quadruplicatum, a rod shaped organism, was grown on glucose at light intensities too low to sustain photosynthesis and CO₂ fixation (24). Chlorogloea fritchii was grown in the dark on acetate (12), a substrate not generally catabolized by blue-green algae (5,21,14). Also, screening of the unicellular culture collection previously maintained at the University of California, revealed six strains, all cocci, capable of photoheterotrophic or chemoheterotrophic growth using glucose as the substrate (19).

One of these strains, Aphanocapsa 6714, was chosen for further study. CO₂ fixation was found to occur solely by the reductive pentose phosphate (Calvin) cycle, while heterotrophic metabolism was carried out predominantly or exclusively by the oxidative pentose phosphate cycle (16,17). In addition, it was found that photosynthesis almost completely suppressed the oxidative pathway. Analysis of enzymes in cell-free extracts revealed that ribulose-1,5-diphosphate (RuDP) was a strong inhibitor of glucose-6-phosphate dehydrogenase, the first enzyme of the oxidative pathway. Moreover, dark incubation of cells led to the immediate disappearance of this metabolite, at the same time the oxidative pathway was being activated. Thus, it was proposed that the intracellular concentration of RuDP acted as a negative control over the oxidation of glucose-6-phosphate, and the oxidative pentose pathway.

In the present study photoautotrophic metabolism of CO₂ is compared with three kinds of glucose metabolism: (1) mixotrophic metabolism of

glucose in photosynthesizing cells, (2) photoheterotrophic metabolism of glucose in cells unable to reduce CO₂ photochemically, and (3) chemoheterotrophic metabolism of glucose in the dark.

In the first condition, cells can carry out both photoautotrophic CO₂ fixation and photophosphorylation along with non-cyclic and cyclic electron photoelectron transport. In condition (2) cells are poisoned with dichlorophenylmethylurea (DCMU) which specifically blocks non-cyclic electron transport and photosynthetic pyridine nucleotide reduction, but leaves ATP synthesis by cyclic phosphorylation undamaged. In condition (3) cells must supply both energy (ATP) and reducing power (NAD(P)H) by dissimilation of glucose.

MATERIALS AND METHODS

Organism: Aphanocapsa 6714 is a unicellular coccus of typological group IIA (22).

Media and Culture Conditions: Cells were grown photoautotrophically in an inorganic medium BG-11, described elsewhere (21). Cells were gassed with compressed air containing about 0.04% CO₂ w/v.

¹⁴C Tracer Experiments: Log-phase cells were harvested at room temperature by centrifugation at 29,000 g for 20 min and resuspended in BG-11 at a concentration of 2% hard packed cells (wet weight) w/v. The cells were then transferred to round bottom flasks (10 ml per flask) sealed with serum stoppers and placed in a rack which suspended the flasks in a water bath kept at 25°C. Agitation was achieved through a reciprocating action of the rack which was attached to an electric motor by a metal connecting rod. The light sources were eight 20-watt Sunray colorite reflectors, mounted about 1 inch below the transparent plexiglass bottom

of the water bath. The light intensity at the surface of the flasks was about 800 foot candles. Light was excluded from dark cells by wrapping a flask with black, waterproof electricians tape. Before starting the experiment, DCMU was added to one of the flasks at a final concentration of 5×10^{-5} M, sufficient to completely suppress O_2 evolution.

At the beginning of experiments, glucose-U- ^{14}C (3.33 mM final concentration; 20 ncuries per nmole) and (or) ^{14}C -sodium bicarbonate (50 mM final concentration; 20.73 ncuries per nmole) were added to each flask by syringe through serum stoppers. To measure the effect of glucose on intermediary metabolism in photosynthesizing cells, both labeled compounds were added to the system to prevent dilution of ^{14}C by fixation of unlabeled CO_2 . When labeled bicarbonate was omitted from cells incubated in the light plus DCMU, or in the dark, there was little difference in pool sizes or polymerized products of metabolism, indicating that virtually all carbon was assimilated from glucose under these conditions.

At intervals, 0.5 ml samples of the cell suspensions were removed by syringe and the cells killed by the addition of 2.0 ml of methanol. From each sample 250 μ l were removed and analyzed by two-dimensional paper chromatography (15).

The solvent system for the first dimension was phenol-water-glacial acetic acid ethylenediamine tetraacetic acid (1 M) (840:160:10:1 v/v), and in the second dimension equal volumes of n-butanol-water (370:25 v/v) and propionic acid-water (180:220 v/v). Compounds in the sugar monophosphate and diphosphate areas of the chromatograms were resolved by development for 48 hr in each direction. Amino acids and PGA were resolved by 24 hr chromatography in each direction. Sugar diphosphates, which migrate as a single spot were re-eluted from chromatograms with distilled water,

hydrolyzed with a partially purified enzyme from a Polidase-S preparation (Schwartz Biochemicals, Inc.), and rechromatographed in the solvent systems described above, 24 and 16 hr respectively. This procedure separated ribulose, sedoheptulose, and fructose as the free sugars.

Radioactive areas on the chromatograms were identified by X-ray film, the spots corresponding to exposed film cut out and counted by an automatic spot-counting device (13). After corrections were made for counting efficiencies and coincident counting, the results were expressed in concentration units of natoms input carbon per mg (wet weight) cells.

Analysis of ^{14}C in Glycogen and Protein: Methanol suspensions used for resolution of soluble intermediates were concentrated by centrifugation and the supernatant containing soluble intermediates discarded. The cell pellets were washed exhaustively, first with 100 mM ^{12}C glucose (if labeled glucose was a substrate) and then with distilled water. The washed pellets were suspended in 2 ml of 2 N HCl in small test tubes, covered with marbles, and hydrolyzed for 1 hr at 100°C. Fifty μl of the supernatant fraction from the hydrolysate was added to Aquasol (New England Nuclear) and counted by liquid scintillation. The remaining supernatant was concentrated to dryness in a vacuum dessicator and then resuspended in 2 ml of 50 mM sodium carbonate to neutralize residual acid. Two hundred fifty μl of this solution was chromatographed in the standard solvent systems as described above, and freed radioactivity determined by radioautography. Unlabeled glucose was added to duplicate chromatograms and the position determined after chromatography by AgNO_3 reagent (23). Approximately 95% of the solubilized radioactivity migrated with free glucose.

The acid hydrolyzed pellets were washed exhaustively as described above, and suspended in 2 ml of an aqueous solution containing 50 μ g of Pronase (Sigma Biochemical Corp.) per ml. 0.10 ml of toluene was layered over the pronase suspension and the two phase system incubated at 37°C for 24 hr. The samples were clarified by centrifugation and 50 μ l of the supernate, containing solubilized amino acids, counted in Aquasol by liquid scintillation.

RESULTS

In Figures 1-3, the ^{14}C labeling of various metabolites is shown for photosynthesis with $^{14}\text{CO}_2$ (A), photosynthesis with $^{14}\text{CO}_2$ and ^{14}C -glucose (B), photoheterotrophic assimilation of ^{14}C -glucose (C), and chemoheterotrophic assimilation of ^{14}C -glucose.

The presence of ^{14}C -labeled glucose during photosynthesis resulted in a 15-fold increase in the concentrations of the sugar monophosphates, glucose-6-phosphate (G6P) and sedoheptulose-7-phosphate (S7P) (shown together as a single curve) and fructose-6-phosphate (F6P). In dark cells the increase was even more dramatic, with approximately a 140-fold increase in the concentrations of these compounds (Figure 1-D). The concentrations of the monophosphates in DCMU-treated cells was intermediate between photosynthetic and dark values (Figure 1-C).

The formation of ^{14}C labeled PGA is about the same during photosynthesis plus and minus glucose, as expected for the primary produce of CO_2 fixation. In DCMU-treated cells the concentration of PGA is reduced to about 50% that found in photosynthetic cells. However, the rate that labeled carbon accumulates in PGA is markedly reduced in dark cells and is barely detectable during the first 15 min of incubation.

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Nearly the same concentrations of ribulose-1,5-diphosphate (RuDP) are maintained in photosynthesizing cells, plus and minus glucose (Figure 2-A, 2-B), but only traces of fructose-1,6-diphosphate (FDP) and sedoheptulose-1,7-diphosphate (SDP) were found. On the other hand, inhibition of photosystem II (non-cyclic electron flow) or dark incubation both lead to the virtual disappearance of ^{14}C in RuDP, while levels of FDP and SDP are elevated many times over the photosynthetic values (Figure 2-C, 2-D).

Phosphogluconate (6-PGluc) was not detectable in the light when $^{14}\text{CO}_2$ was used as the only carbon source (Figure 2-A), but a slow labeling of this metabolite was noted in cells assimilating ^{14}C -glucose in the light (Figure 2-B). On the other hand, a comparatively large increase in labeled 6-PGluc is seen in dark cells (Figure 2-D), probably as a result of the active use of the oxidative pentose pathway. Cells treated with DCMU, in the light, showed a faster rate of ^{14}C accumulation in this metabolite than photosynthetic cells (Figure 2-C), but slower than dark cells.

Incorporation of labeled carbon into glutamate and aspartate was not greatly different in photosynthetic and DCMU treated cells, except for the early saturation of the aspartate pool in $^{14}\text{CO}_2$ -fed cells (Figure 3-A, 3-B, 3-C). In contrast to these results, dark cells took up little ^{14}C into aspartate and the rate of glutamate labeling was only 15-30% that for the other cells (Figure 3-D).

Polymer Synthesis: Figures 4-A and 4-B show the net synthesis of protein and glycogen (polyglucose) by the cells of Figures 1-3. A large amount of glucose was incorporated into glycogen in all glucose-fed cells. The rate in dark cells was approximately 1/2 that observed in photosynthetic or cells treated with DCMU in the light.

Rates of protein synthesis were highest in photosynthetic cells, lowest in dark cells, and intermediate in cells treated with DCMU. It is noteworthy that the rates of glycogen and protein synthesis were more nearly comparable in CO₂ fed cells--thus, glucose enhances the formation of glycogen over the synthesis of protein.

Dilution Experiments: Replacement of ¹⁴C by an excess of unlabeled carbon from glucose and (or) CO₂ is shown in Figures 5 and 6. Dilution rates were rapid in photosynthetic cells, with half times of about 1 min for the three monophosphates and RuDP (Figures 5-A, 6-A). The corresponding dilution rates from dark cells were much slower: 10-12 min for monophosphates, and approximately 8 min for diphosphates (Figures 5-C, 6-C). The rates in DCMU-treated cells were about 2-3 min for the monophosphates and diphosphates (Figures 5-B, 6-B).

DISCUSSION

Inhibition of photosystem II (hence of non-cyclic electron flow) or dark incubation leads to activation of the oxidative pentose pathway and loss of reductive carboxylation. Thus, non-cyclic electron flow may exert the primary control over intermediary metabolism in Aphanocapsa 6714. A target site for control of the reductive pentose phosphate (Calvin) cycle is phosphoribulokinase, since RuDP is not synthesized in the dark, or in the light plus DCMU. Also, previous results have shown the rapid disappearance of this metabolite in cells transferred to the dark (16). This indicates inactivation of the kinase reaction although carboxydismutase continues to catalyze the formation of PGA from RuDP and CO₂, at least during the first several minutes. It should be noted that the phosphoribulokinase from spinach chloroplasts is also inactivated in the dark,

but can be reactivated, in vitro, by light in the presence of chloroplasts, or in the dark by reduced dithiothreitol (9). This suggests that reduction of a critical site on the enzyme is necessary for activity. In this regard, it should also be noted that NADH is a positive effector of the phosphoribulokinases from the photosynthetic bacterium, Rhodospseudomonas spheroides and the facultative autotroph, Hydrogenomonas facilis (18,11). AMP is a potent inhibitor of these enzymes, as well as the enzymes from the two thiobacilli, T. thiooxidans and T. thioparus (10,6). Phosphoribulokinase from the thiobacilli is apparently unaffected by NADH.

The loss of non-cyclic electron flow due to darkness or DCMU inhibition in the light also has important effects on the ease with which carbon moves from the intermediates of the pentose phosphate pathways to the synthesis of amino acids. This can be seen in two ways. The rate at which the pools of amino acids and phosphoglycerate are built up in dark cells is much slower than in photosynthetic cells, and rates of protein synthesis in dark and DCMU-treated cells are reduced by more than 80% and about 50% respectively. Second, the turnover rates of metabolites in the monophosphates and diphosphates are reduced by about the same amounts (80 and 50%) in dark and in DCMU-treated cells in comparison to photosynthetic cells. It is clear that the loss of photosystem II activity results in a substantial reduction in the overall rate of metabolism.

This reduced metabolic rate is probably not due to energy limitation since dark cells contain as high, or higher, levels of ATP as photosynthetic cells (paper in preparation). Rather, we feel the cause is likely to be a decrease in the capability of regulated enzymes to catalyze key reactions. One of these reactions is probably the hydrolysis

of fructose-1,6-diphosphate. That there is a decrease in the activity of this enzyme can be seen by the increase in concentration of fructose-1,6-diphosphate (along with sedoheptulose-1,7-diphosphate) in dark and DCMU-treated cells. Since the hydrolysis of fructose-1,6-diphosphate to fructose-6-phosphate (which may be catalyzed by the same enzyme in Chlorella and higher plants) is strongly favored in photosynthesizing cells, the increased concentration of fructose-1,6-diphosphate in dark cells and cells treated with DCMU, in the light, means that the enzyme involved fructose-1,6-diphosphatase (FDPase) is functioning at a slower rate.

In Chlorella, FDPase activity greatly decreases in the dark, or in the light with the addition of vitamin K₅, thought to divert electrons from non-cyclic photoelectron flow (15,8). The resulting oxidized state of cofactors in the chloroplast is thought to activate glucose-6-phosphate dehydrogenase and the oxidative pentose phosphate cycle as well (1,7). The oxidative pentose pathway still operates recycling triose phosphates via FDPase to glucose-6-phosphate, although the enzyme's activity is greatly reduced. Thus, the overall features of regulation between the oxidative and reductive pentose pathways in Aphanocapsa 6714 resemble those of the more well studied system, Chlorella pyrenoidosa.

The potential for heterotrophic growth by Aphanocapsa 6714 is probably due to the permeability of the cell membrane to glucose, rather than to a fundamental biochemical difference between this organism and the strictly photoautotrophic blue-green algae (17). In all of the organisms that we studied, glycogen was the major reserve material of the cell, the levels of the glucose-6-phosphate and gluconate-6-phosphate dehydrogenases were comparatively high and the activity of the latter enzyme was immediately

evident on a transition of cell suspensions from light to darkness (16). Thus it seems reasonable to expect that the oxidative pentose cycle is used for endogenous metabolism by these organisms. In resting cells only a limited flow of carbon through the cycle sufficient for maintenance levels of ATP would be required. Accordingly, regulation of the phosphatase reaction converting fructose-1,6-diphosphate to fructose-6-phosphate could serve as one mechanism for reducing the activity of the oxidative cycle to match the catabolic requirements of the cell. With the exception of the FDPase reaction, the other steps between triose phosphate and glucose-6-phosphate are known to be reversible in photosynthetic systems (2). It is noteworthy that the spinach enzyme is dependent on a strong reducing agent such as ferredoxin for maximum activity (3) and that low potential electron donors more negative than the pyridine reduced nucleotides are unlikely to exist in plant-type photosynthetic systems in the absence of photoelectron flow.

It has already been shown that the rate of protein synthesis and formation of amino acid precursors is more strongly inhibited than the synthesis of glycogen in dark cells of Aphanocapsa 6714. Thus, recycling of a significant portion of triose phosphate generated by the oxidative pentose cycle is required. Accordingly, we would look for other enzymes between triose phosphate and the tricarboxylic acid cycle reactions also to undergo inactivation in the dark. There are a number of possible candidates to be found in the literature on enzyme regulation in photosynthetic systems: the NADP⁺ linked triose phosphate dehydrogenase (24,20), phosphopyruvate carboxylase (4), and pyruvate kinase (7).

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FIGURE CAPTIONS

Fig. 1. ^{14}C -labeled intermediates in Aphanocapsa 6714 under four experimental conditions: (A) light (as energy source), $^{14}\text{CO}_2$ as carbon source; (B) light, $^{14}\text{CO}_2$ and ^{14}C -glucose as carbon sources; (C) light with 5×10^{-5} M DCMU to block non-cyclic photoelectron flow and ^{14}C -glucose as carbon source; (D) darkness with ^{14}C -glucose as carbon and energy source. 3-phosphoglycerate, o; monophosphates (G6P and S7P), ●; F6P, ▩.

Fig. 2. ^{14}C -labeled intermediates in Aphanocapsa 6714 under four experimental conditions (see Fig. 1). RuDP, ●; FDP, ▩; SDP, Δ; 6-phosphogluconate, o.

Fig. 3. ^{14}C -labeled intermediates in Aphanocapsa 6714 under four experimental conditions (see Fig. 1). Glutamate, o; aspartate, ●.

Fig. 4. Incorporation of ^{14}C into glycogen (polyglucose) and protein from $^{14}\text{CO}_2$ and ^{14}C -glucose.

(A) Protein synthesis in cells incubated with:

●, light (as energy source), $^{14}\text{CO}_2$ and ^{14}C -glucose as carbon sources.

Δ, light plus $^{14}\text{CO}_2$ as carbon source.

o, light plus 5×10^{-5} M DCMU (to inhibit non-cyclic photoelectron flow) and ^{14}C -glucose as carbon source.

Δ, darkness with ^{14}C -glucose as carbon and energy source.

(B) Glycogen synthesis in cells incubated as described in (A).

Fig. 5. Dilution of ^{14}C from intermediates of Aphanocapsa 6714.

- (A) Light, ^{14}C -glucose and $^{14}\text{CO}_2$ as carbon sources. At 10 min (dashed line), 100: 1 excess of ^{12}C -glucose and bicarbonate added to the system.
- (B) Light with 5×10^{-5} M DCMU, ^{14}C -glucose as carbon source. At 10 min 100: 1 excess of ^{12}C -glucose added to the system.
- (C) Darkness, ^{14}C -glucose as carbon and energy source. At 10 min 100: 1 excess of ^{12}C -glucose added to system.

Monophosphates (G6P and S7P), ●; F6P, ○.

Fig. 6. Dilution of ^{14}C from intermediates of Aphanocapsa 6714

(see Fig. 5 for explanation of conditions (A)-(C)).

RuDP, ●; FDP and SDP, ○.

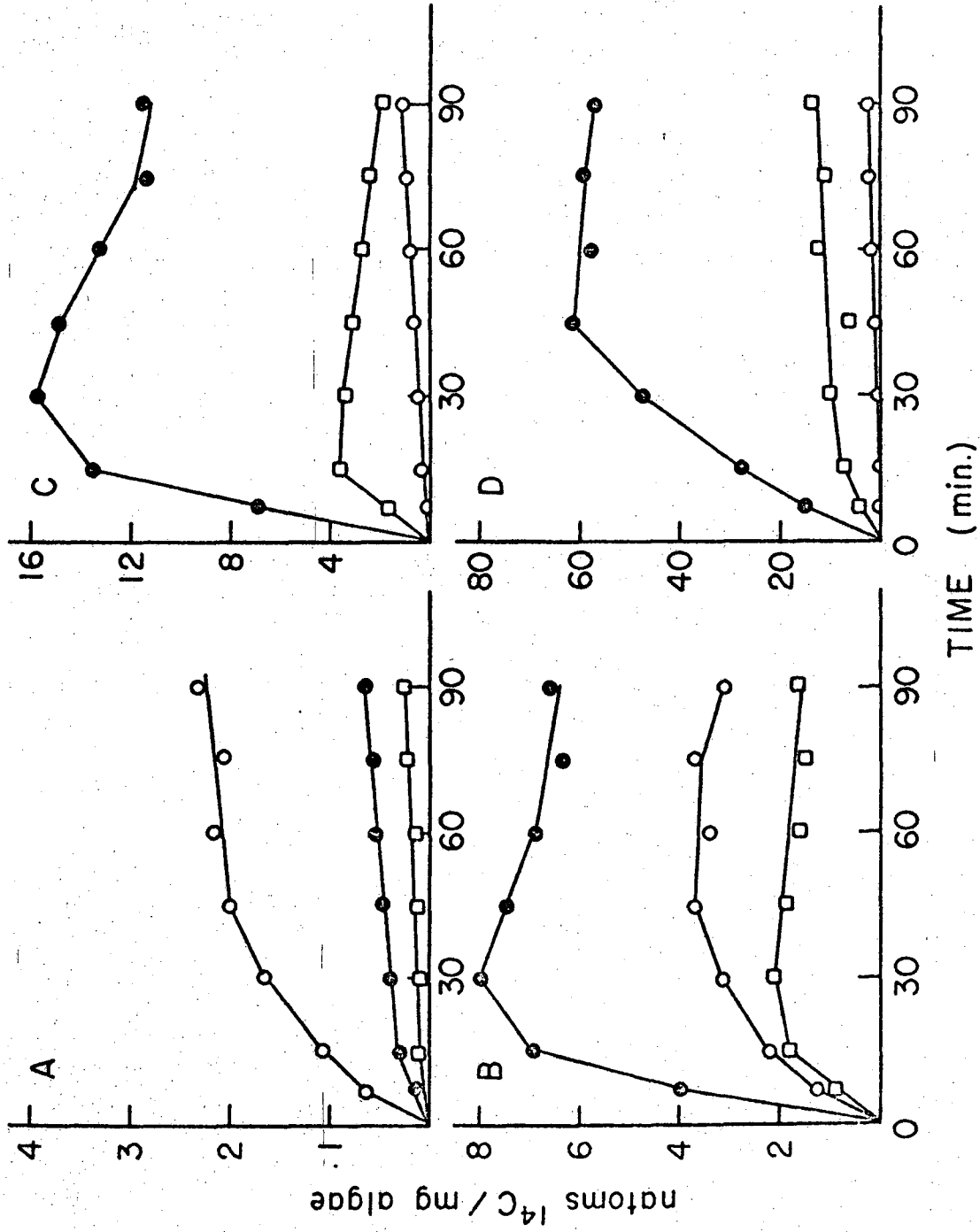


Fig 1. Release of ¹⁴C from algae

XBL7212-4823

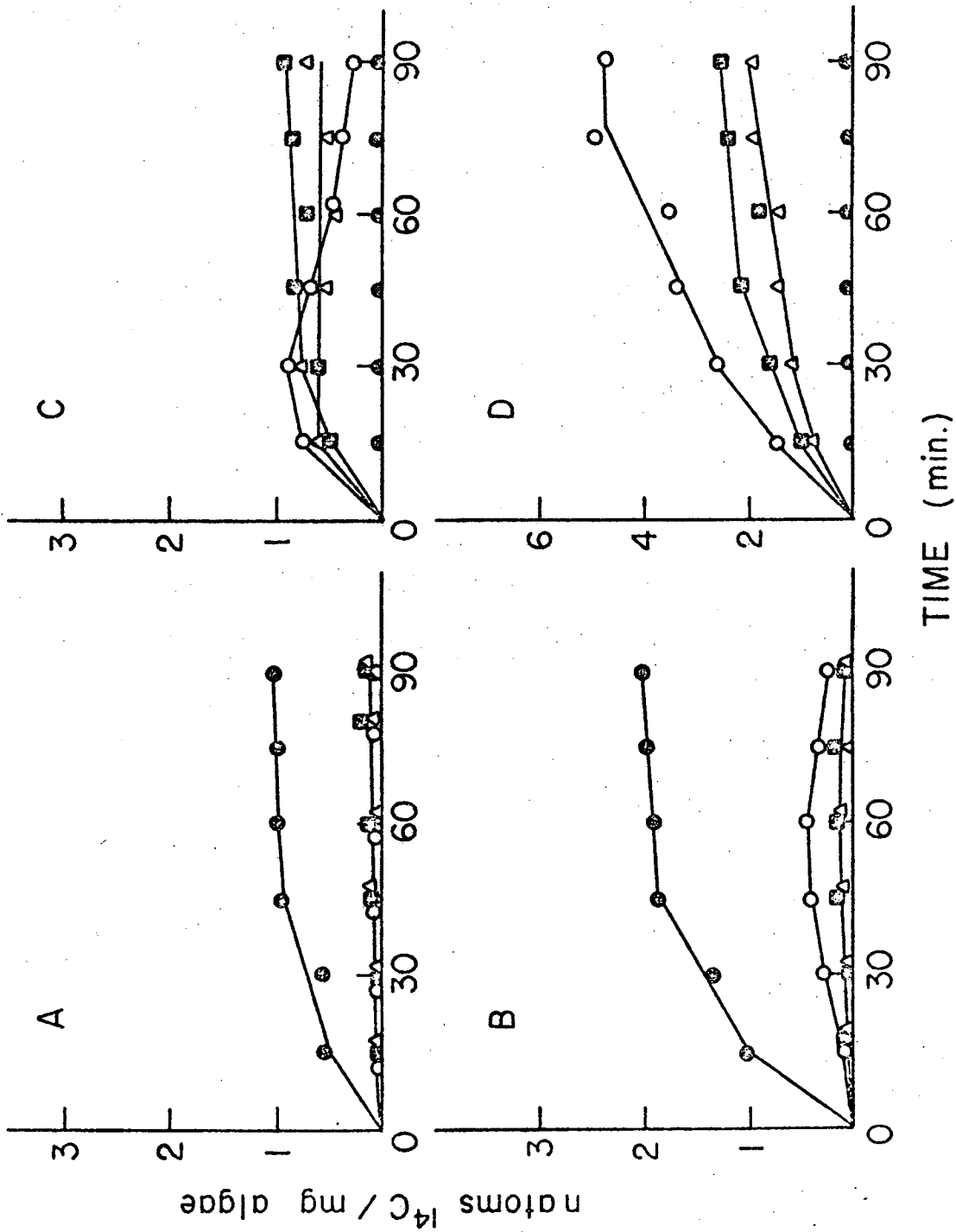
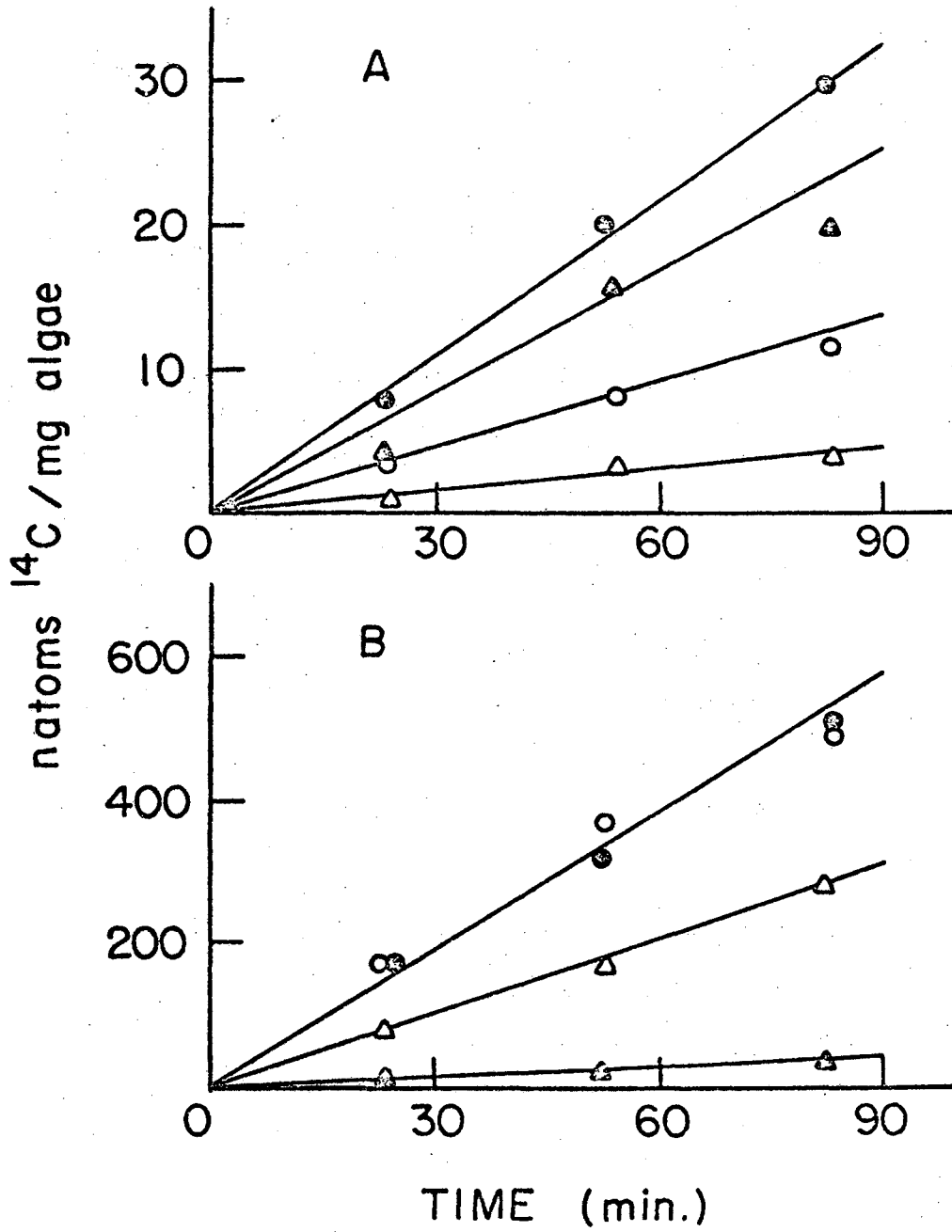


Fig 2 Release of ¹⁴C from algae

XBL7212-4825



XBL7212-4826

Fig 4 Jeffrey & Posthum

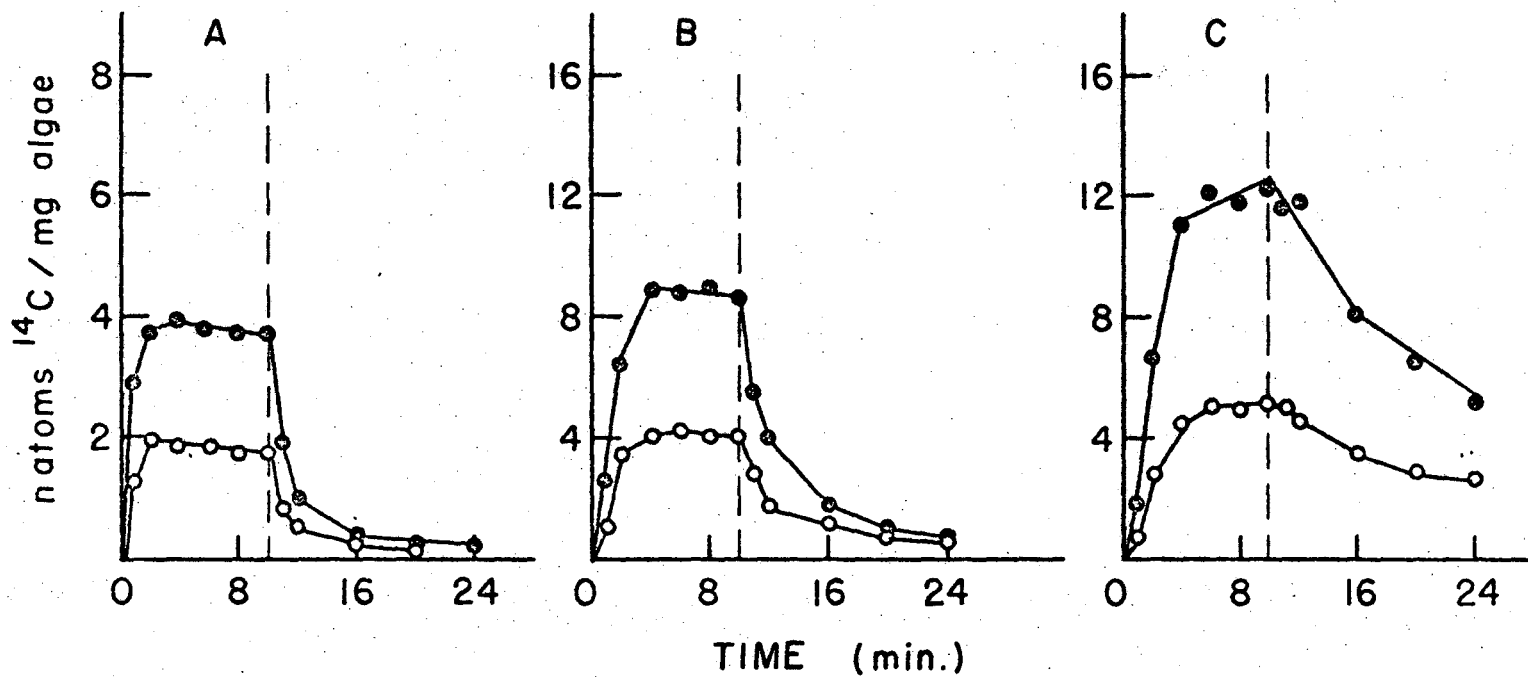
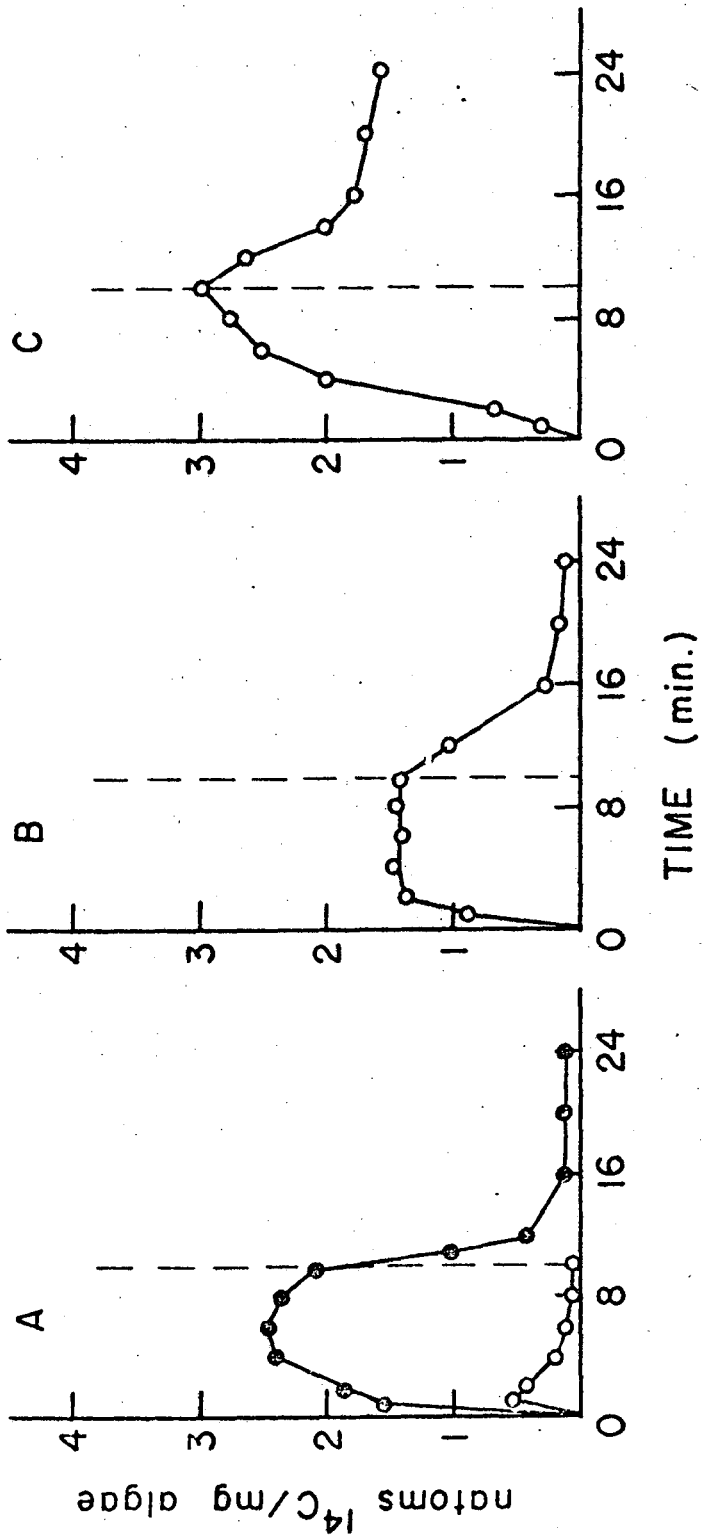


Fig 5 Pelroy & Bassham

XBL7212-4828



XBL 7212-4827

Fig 6 Polunin & Bassham

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