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Author
Paul, John S.

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John S. Paul and James A. Bassham

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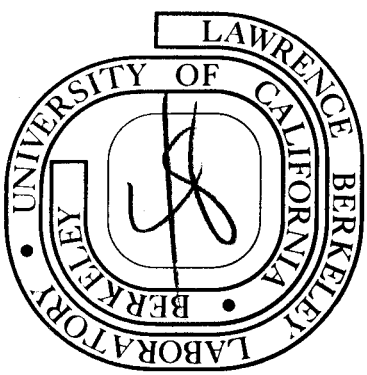

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DISCLAIMER

Running Title: Sulfite Effects on Mesophyll Cells

Mailing Address:

John S. Paul

Laboartory of Chemical Biodynamics

Lawrence Berkeley Laboratory

University of California

Berkeley, California 94720

EFFECTS OF SULFITE ON METABOLISM IN ISOLATED MESOPHYLL CELLS FROM *PAPAVER SOMNIFERUM*

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John S. Paul and James A. Bassham

Laboratory of Chemical Biodynamics

Lawrence Berkeley Laboratory

University of California

Berkeley, California 94720

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

[2] Abbreviations: OAA: oxalacetate; PGA: 3-phosphoglycerate; PVP: polyvinyl pyrrolidone; TCA; tricarboxylic acid.

ABSTRACT

Brief exposure (30 min) of leaf-free mesophyll cells from the C-3 plant, Papaver somniferum to concentrations of sulfite (SO_2 + HSO_3^- + $SO_3^=$) up to 20 mM stimulated the rate of CO_2 incorporation as much as 30%. In addition, the sulfite rapidly affects the metabolism of newly incorporated CO_2. Ammonia incorporation into glutamine and subsequent transamination reactions were stimulated during the short-term exposure periods while glycolate metabolism apparently was inhibited by bisulfite at two points in the pathway. In addition, the results indicate that glycolate is the major precursor of glycine in these cells. Prolonged periods of exposure (24 h) to sulfite had somewhat different effects on carbon metabolism: the high concentrations (10-20 mM) severely inhibited all aspects of cellular metabolism while lower concentrations (1 mM) appeared to inhibit ammonia incorporation but stimulated synthesis of sucrose and starch.

The effects of the air pollutant, SO_2, on plant cell metabolism are of increasing interest given the growing dependence of industrialized nations on combustion of high sulfur fuels for energy and consequent increased emissions of SO_2 to the atmosphere. Numberous studies have been made in recent years on both morphological and biochemical effects (see 1, 19). Unfortunately the results are often contradictory, probably due to the influence of other factors such as light, temperature, humidity, and period of exposure. Effects of SO_2 on whole plants or leaves include effects on overall gas exchange into the leaf (7, 18). Given the possibilities for dissolved SO_2 to react chemically as an acid as well as a bisulfite ion at various morphological and biochemical points in leaves and cells, it is desirable to sort out these reactivities with a simpler system.

The isolation and maintenance in liquid suspension of photosynthetically viable mesophyll cells from a C-3 plant (8) offers an excellent system in which to study biochemical perturbations by sulfite ($SO_2 + HSO_3^- + SO_3^=$) on plant cell metabolism. Not only have the morphological restraints of the leaves been removed, but the cellular environment can be more accurately maintained and described. With such a system it is possible to evaluate the effects of SO_2 at different concentrations and lengths of exposure. Such information could be useful in estimating the susceptibility of plants to damage or even to possible beneficial effects of SO_2 under various physiological conditions.

MATERIALS AND METHODS

Cell Isolation. Mesophyll cells were isolated from a C3 plant, Papaver somniferum, using the enzyme pectinase as previously described (8). Digestion medium (pH 5.7) and assay medium (pH 8.0) used were also as previously described except that bovine serum albumin was brought to 0.2% (w/v) in both and PVP was included at a concentration of 0.1% (w/v).

Cells were maintained for 24 h in assay medium (12 h dark, 8°C 12 h light, 24°C) before commencing the assays. Thus control cells in the assays reflected the metabolic state of mesophyll cells in the leaf (8). In a second experiment, part of the cells were maintained for 24 h in the presence of various concentrations of sulfite so that a comparison of short-term and long-term effects of sulfite could be made.

Photosynthetic $^{14}CO_2$ Incorporation. 1500 µl aliquots of the cell suspensions were placed in serum stoppered micro-fernbach flasks and assayed at 22°C in a Plexiglas water bath illuminated from below with flourescent lamps (450 $\mu E \cdot M^{-2} \cdot sec^{-1}$)(5). The flasks were attached through manifolds to a steady-state gas circulation system (11). After incubating the cells for a few minutes in the light in CO_2-free air, the experiment was started with the addition of 380 ppm $^{14}CO_2$ (14 µCi/mole) in air. For assessing the short-term effects of sulfite on metabolism, cells were incubated with $^{14}CO_2$ for 30 min; sulfite was then added to some flasks at a final concentration of either 10 mM or 20 mM (HSO_3^- + $SO_3^=$) and the assay continued for an additional 30 min. 100 µl samples were taken at 10 min intervals before the addition of sulfite and at 5 min intervals after the addition. All samples were killed immediately in 80%

methanol. The stock solution of sulfite was brought up to pH 8.0 before its addition to the cells.

For assessing the long-term effects of sulfite on metabolism, cells which had been maintained in the peresence of sulfite for 24 h were assayed at 380 ppm $^{14}CO_2$ in air for 60 min. 100 µl samples were taken at 10 min intervals throughout this time period.

Analysis of Photosynthetic Products. Separation and identification of the soluble products of ^{14}C-incorporation was accomplished by descending paper chromatography and radioautography as previously described (8,10). Starch was determined from the insoluble fraction as before (8). Glycine, serine, glycolate and glycerate were separated on a special chromatographic system according to the method of Platt and Bassham (13). All incorporation results are expressed on the basis of µg-atoms of ^{14}C incorporated per mg Chl.

RESULTS

Short-term Effects of Sulfite. Addition of either 10 mM or 20 mM sulfite (HSO_3^- + $SO_3^=$) to photosynthesizing cells of P. somniferum caused a small but reproducible stimulation of CO_2 incorporation up to 30% (Fig. 1). The increased fixation was found to be almost as high at elevated partial pressures of CO_2 (0.4%) as at air level (0.038%) (Table 1).

Analysis of metabolic products revealed, not surprisingly, that bisulfite stimulated an immediate increase in pools of glycolate and a concomitant decrease in the glycine pool (Fig. 2). Somewhat unexpected, however, was another apparent effect of bisulfite on the Tolbert pathway (17) of glycolate metabolism: the serine pool displayed a transient

increase while, at the same time, a precipitous drop in glycerate occurred (Fig. 3). After the serine pool had nearly doubled in size, the level of glycerate again started to rise.

In addition to glycolate metabolism, the flow of carbon through the TCA Cycle (Fig. 4) and into glutamate and glutamine (Fig. 5) was also affected by sulfite. The nature of the stimulation seen is reminiscent of the effect of ammonia on photosynthetic carbon metabolism (9) particularly with the observed sharp increases in aspartate and alanine (Fig. 6). Significant increases in the labeling rates of sucrose and starch were also seen (Fig. 7). These tend to parallel the increase in overall incorporation rate (Fig. 1).

Long-term Exposure to Sulfite. Cells incubated with 1 mM sulfite for 24 h (12 h dark, 12 h light) showed only a slight stimulation of CO_2 incorporation and a very different incorporation pattern when compared to either control cells or those exposed for transient periods to sulfite (Table 2). Particularly notable is the impairment of TCA Cycle labeling as well as that of related amino acids (aspartate and glutamine) with the exception of glutamate. Glycolate metabolism appears to be unimpaired at this concentration of sulfite (1 mM) while the flux of carbon into the storage saccharides remained higher than in control cells. Concentrations of sulfite which were stimulatory in the short-term kinetic experiments (10 mM and 20 mM) were found to be quite inhibitory after 24 h of exposure. The indicated permeability problems are discussed below.

DISCUSSION

Short-term Effects. The stimulation of CO_2 incorporation by sulfite (HSO_3^- + $SO_3^=$) can only be partially accounted for by blockage of glycolate

metabolism since the increased fixation is nearly as high at the elevated levels of CO_2 as at air level (Table 1). A comparable stimulation of photosynthesis in isolated chloroplasts has also been noted in the presence of up to 1 mM HSO_3^- (6). No mechanism has been proposed.

In addition to effects on overall CO_2 incorporation, interaction of bisulfite with glycolate metabolism was noted. The results indicate that at air level concentrations of CO_2, glycolate formation is largely responsible for glycine biosynthesis (Fig. 2). The formation of a glyoxylate bisulfite addition compound is presumed to occur and this compound may then competitively inhibit the glycolate oxidase responsible for converting glycolate to glyoxylate. The increase in labeled glycolate during the 10 min following bisulfite addition is only about one-fourth the decrease in labeled glycine. These kinetics could be explained if there are small, actively turning over pools of glycolate and glyoxylate, with only partial inhibition of the oxidation of glycolate to glyoxylate. The decreased rate of formation of glyoxylate, together with the conversion of glyoxylate to the bisulfite addition compound could result in a drop in the glyoxylate level (which we did not measure) to well below the K_m for glyoxylate of the transaminase. When glycolate reached a high enough concentration to allow it to compete effectively with the inhibitor, the glycine pool began to recover.

The competitive inhibition of glycolate oxidase by the glyoxylate bisulfite addition compound apparently is not the only point of interaction between bisulfite and the glycolate pathway. The transient rise in serine and the immediate and precipitous drop in glycerate (Fig. 3) are indicative of at least a partial block between those two compounds. We propose that

bisulfite could form an addition compound with hydroxypyruvate in a manner similar to that with glyoxylate (2) and this compound, in turn, could be playing the role of a competitive inhibitor perhaps at the oxidation step (hydroxypyruvate to glycerate) (Fig. 8). The transient increase in serine would be the result of continuing metabolism of the existing glycine pool (which drops concomitantly) together with the partial inhibition of serine metabolism. The rapid refilling of the glycerate pool following its initial precipitous drop might reflect an activation of phosphoglycerate phosphatase and, hence, synthesis of glycerate from PGA (15-16). If there is a synthesis of serine possible by two pathways (from glycolate and from 3-phosphoglycerate) as is suggested by this data as well as other work (12, 16), strict metabolic regulation of glycerate kinase and phosphoglycerate phosphatase must be required to avoid a futile cycle.

A further short-term effect of sulfite on mesophyll cell metabolism appears to be an overall stimulation of ammonia incorporation as evidenced by increases in glutamine and glutamate pools together with elevated amination of other amino acids. These results are in agreement with those reported by Jager et al. (4). The rise in TCA Cycle intermediates (citrate and malate) probably reflects increased anaplerotic reactions to supply the glutamine/glutamate pools.

Long-term Effects. After short exposures of the cells to bisulfite, carbon continues to move freely through glycolysis and the TCA cycle to glutamate, alanine and aspartate, respectively. Only after a prolonged exposure to the sulfite anions (HSO_3^- + $SO_3^=$) did an apparent inhibition of labeling of TCA Cycle intermediates develop (Table 2). This may reflect a low permeability of the mitochondria for sulfite species. Of the related

intermediates, only glutamate accumulated faster than in the control cells, perhaps indicating a partial inhibition of glutamine synthetase since glutamine labeling was significantly reduced.

Cells incubated with sulfite for the extended time period were exposed to a much lower concentration (1 mM) than under the short-term incubation (10 mM). This fact is reflected in the apparent absence of perturbation of glycolate metabolism after the prolonged incubation. As mentioned, long exposures to the higher concentrations of sulfite (10 mM or 20 mM) resulted in severe inhibition of all aspects of cellular metabolism.

Conclusion. At first glance, the stimulation of photosynthesis by short-term exposures to sulfite species appears to contradict the results of others. In the review by Ziegler (19), it is mentioned that sulfite always inhibits photosynthesis when present in concentrations exceeding 1 mM. Although 10 mM sulfite -bisulfite was used in the short-term experiments, the concentration of HSO_3^- inside the cells was probably much lower. At the pH used in the assay (pH 8.0) and with a pK_2 = 6.9 most of the sulfite species exists outside the cell as $SO_3^=$ (less than 10% is HSO_3^- while SO_2 is negligable at this pH). The rapidity by which sulfite was seen to affect the flow of photosynthate is an indication that the plasmalemma has a relatively high permeability for at least one of the sulfite species but not necessarily both. If that permeability is only for HSO_3^- then the effective concentration outside the cell would be less than 1 mM instead of 1o mM. In addition, an intracellular pH between 7 and 8 would result in a significant shift of newly acquired HSO_3^- to $SO_3^=$. Under the short-term incubations, organelles such as chloroplasts may thus be exposed to sufficiently small concentrations of the active species of sulfite to allow for a stimulation

of CO_2 fixation. Such a stimulation has been reported with isolated spinach chloroplasts at concentrations of sulfite species up to 1 mM (6). The interpretation of HSO_3^- as the active species is consistent with the findings of Hill (3) and Pickett et al. (14) who determined that sulfite is only inhibitory on lichens at acidic pH's where the bisulfite ion prevails. Prolonged incubations at 10 or 20 mM sulfite ($SO_3^= + HSO_3^-$) would result in increasing concentrations within the cell and eventually the inhibition observed.

ACKNOWLEDGEMENTS

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TABLE 1. Comparison of sulfite effects at high and air level concentrations of CO_2.

	0.03% CO_2	0.40% CO_2
Control	32 (100%)	120 (100%)
10 mM Sulfite	38 (119%)	132 (110%)
20 mM Sulfite	41 (128%)	150 (126%)

Results expressed μmoles CO_2 incorporated per hour per mg Chl.

TABLE 2. Comparison of photosynthate between control cells and cells incubated for 24 h (12 h light/12 h dark) with 1 mM sulfite.

	Control	1 mM HSO_3^- + $SO_3^=$
Incorporation	26 (100)	28 (109)
Malate	1.7 (100)	.45 (26)
Citrate	0.2 (100)	.14 (70)
Aspartate	0.16 (100)	.10 (63)
Glutamine	0.15 (100)	.08 (53)
Glutamate	0.03 (100)	.05 (162)
Alanine	0.17 (100)	.27 (160)
Starch	3.8 (100)	6.1 (161)
Sucrose	5.8 (100)	7.0 (121)
Glycolate	0.08 (100)	.07 (88)
Glycine	1.0 (100)	.85 (85)
Serine	.95 (100)	.92 (97)

Cells were assayed at air level $^{14}CO_2$ (14 μCi/μmole) for 45 min. Results are expressed as μg-atoms C · mg Chl^{-1}. Relative percentages are in parenthesis, control compounds have been arbitrarily set at 100%.

LEGENDS TO FIGURES

Figure 1. Effect of sulfite (HSO_3^- + $SO_3^=$) on net CO_2 incorporation. Cells were allowed to photosynthesize for 30 min in the presence of air level $^{14}CO_2$ before sulfite was added to a final concentration of 10 mM. Incorporation was then monitored for an additional 30 min in the presence (●) and absence (○) of sulfite.

Figure 2. Effect of sulfite (HSO_3^- + $SO_3^=$) on the labeling of glycine (△) and glycolate (○) pools in the isolated mesophyll cells. Treatment of the cells was as described in Fig. 1 with respective pools being monitored in the presence (closed symbols) and absence (open symbols) of sulfite.

Figure 3. Effect of sulfite (HSO_3^- + $SO_3^=$) on the labeling of serine (□) and glycerate (○) pools. Treatment of the cells was as described in Fig. 1. with respective pools being monitored in the presence (closed symbols) and absence (open symbols) of sulfite.

Figure 4. Effect of sulfite (HSO_3^- + $SO_3^=$) on the TCA Cycle intermediates citrate (▽) and malate (○). Treatment of the cells was as described in Fig. 1 with respective pools being monitored in the presence (closed symbols) and absence (open symbols) of sulfite.

Figure 5. Effect of sulfite (HSO_3^- + $SO_3^=$) on the labeling of glutamate (□) and glutamine (○) pools. Treatment of the cells was as described in Fig. 1 with respective pools being monitored in the presence (closed symbols) and absence (open symbols) of sulfite.

Figure 6. Effect of sulfite (HSO_3^- + $SO_3^=$) on the labeling of the amino acids alanine (△) and aspartate (○). Treatment of the cells was as described in Fig. 1 with respective pools being monitored in the presence (closed symbols) and absence (open symbols) of sulfite.

Figure 7. Effect of sulfite (HSO_3^- + $SO_3^=$) on the labeling of the carbohydrates sucrose (△) and starch (○). Treatment of the cells was as described in Fig. 1 with respective pools being monitored in the presence (closed symbols) and absence (open symbols) of sulfite.

Figure 8. Abbreviated scheme of the Tolbert Pathway (17) of glycolate metabolism showing apparent points of inhibition by bisulfite. Inhibition would presumably result from the interaction of α-hydroxysulfonates formed from the appropriate ketoacids.

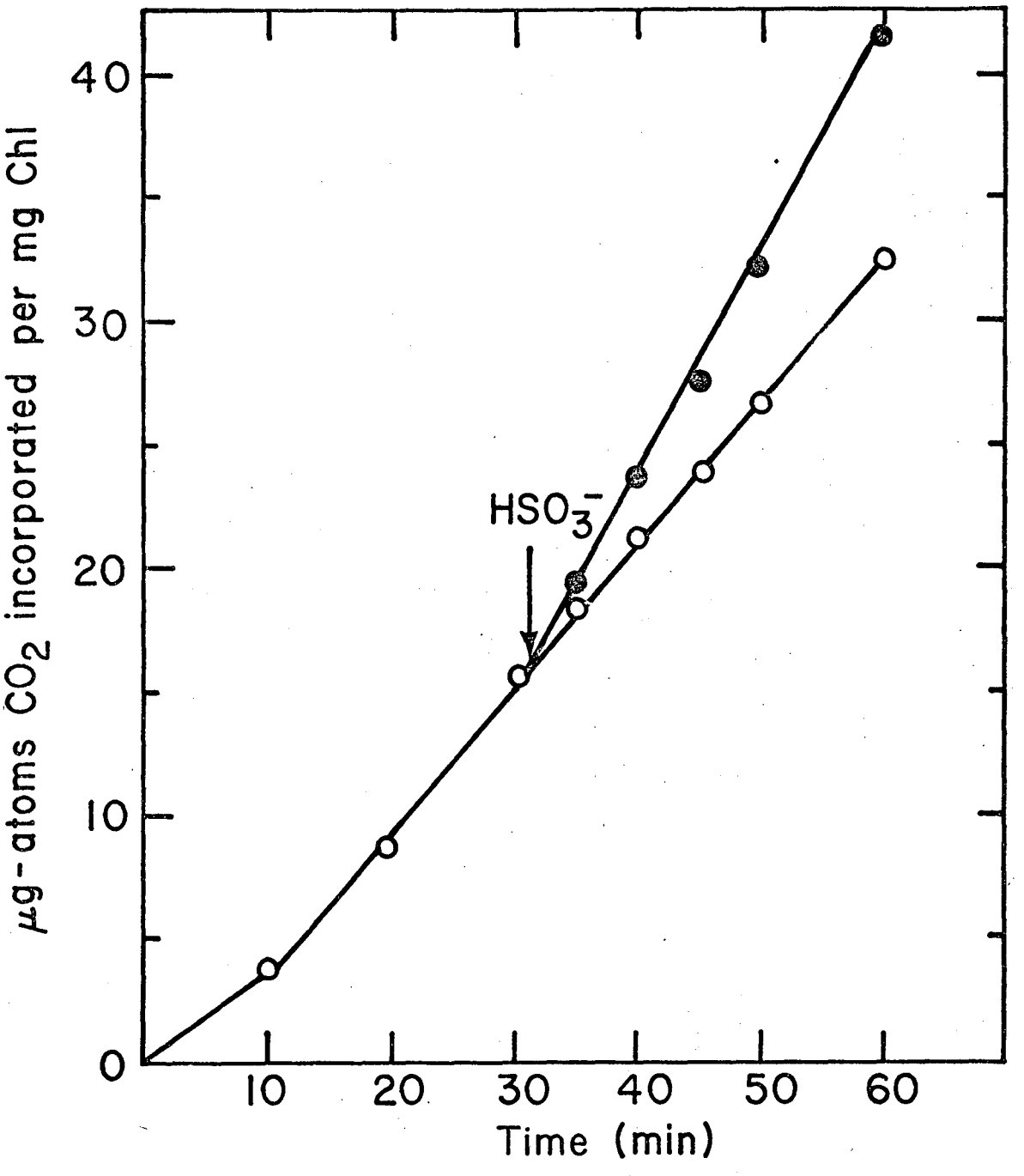

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Fig. 1

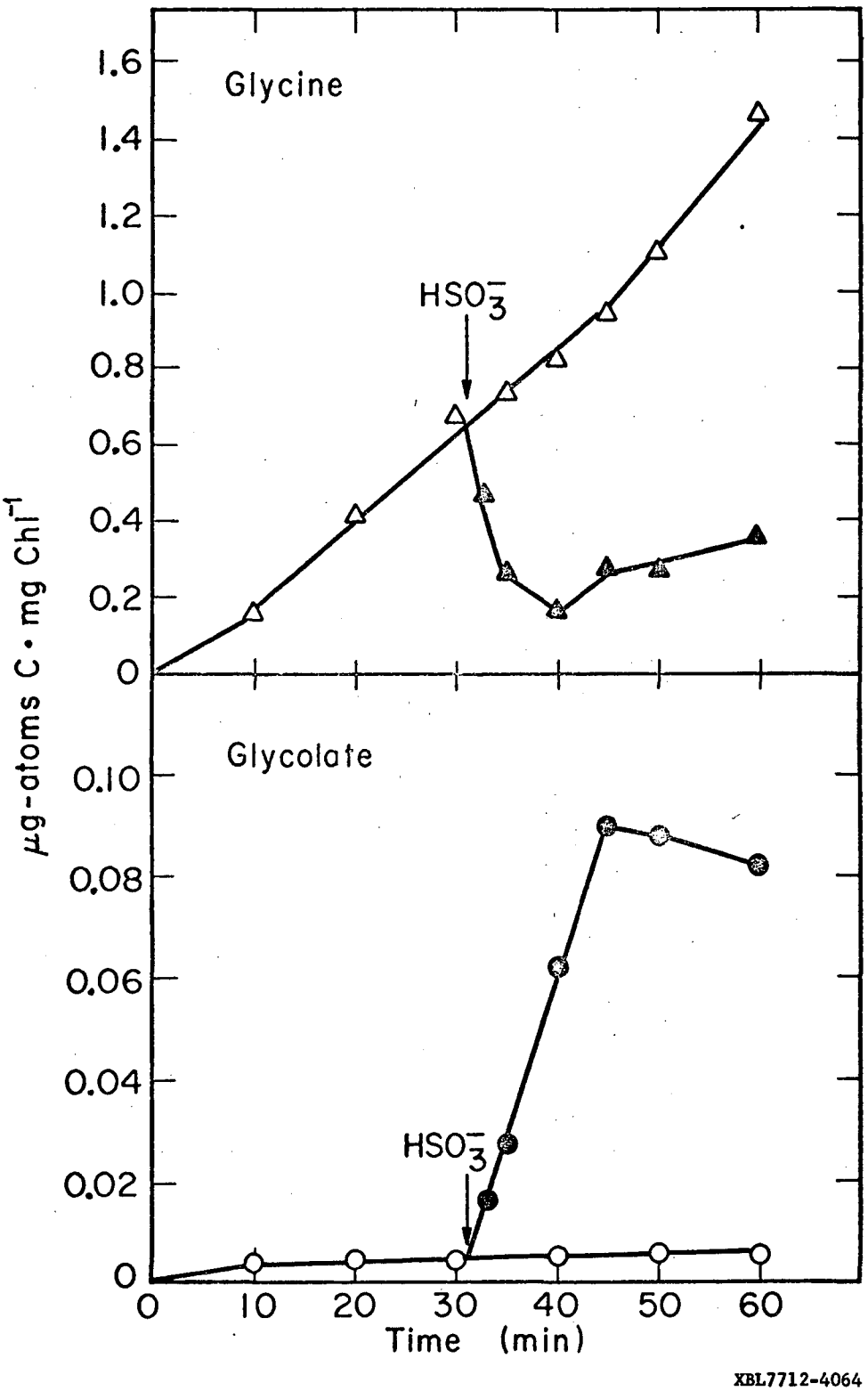

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Fig. 2

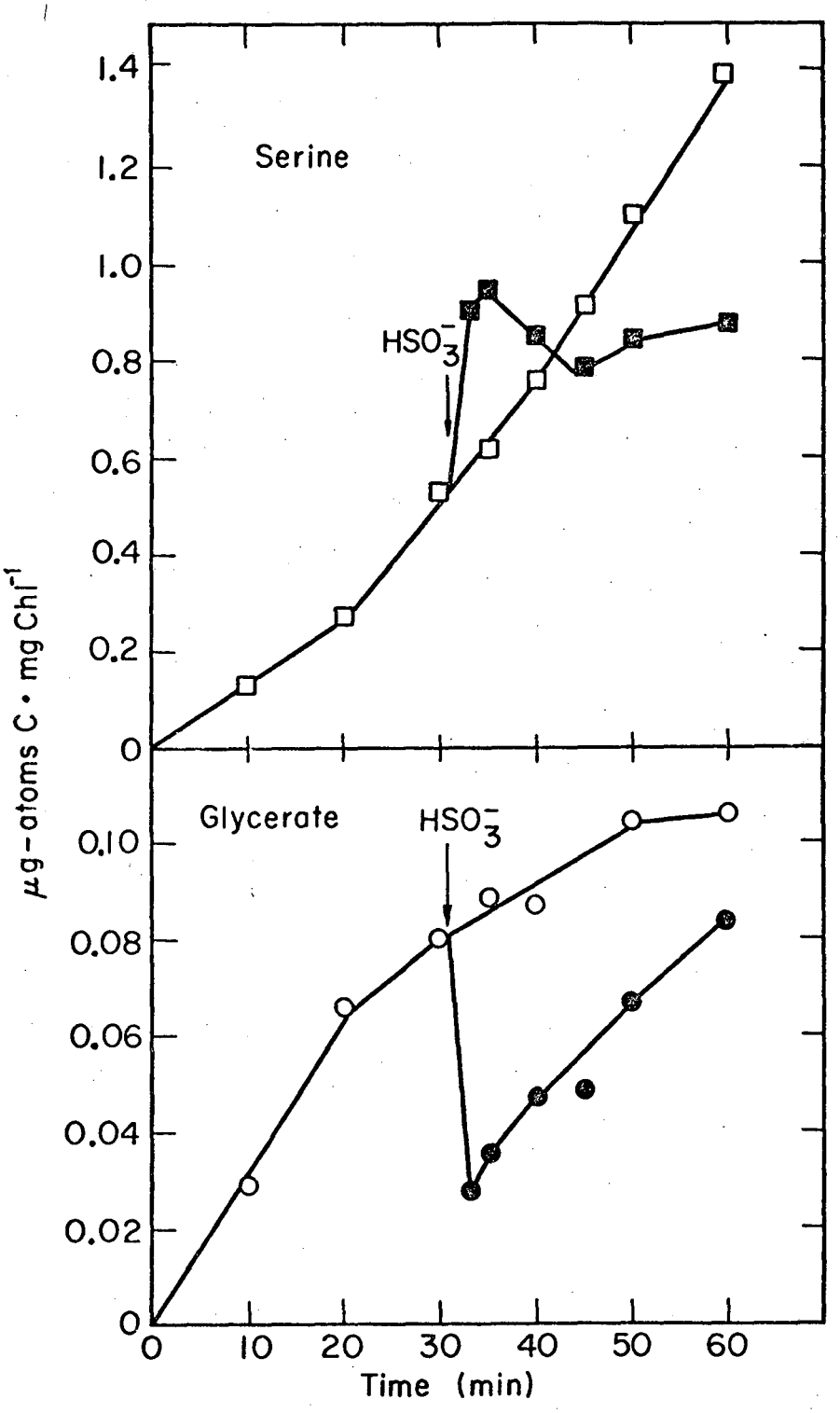

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Fig. 3

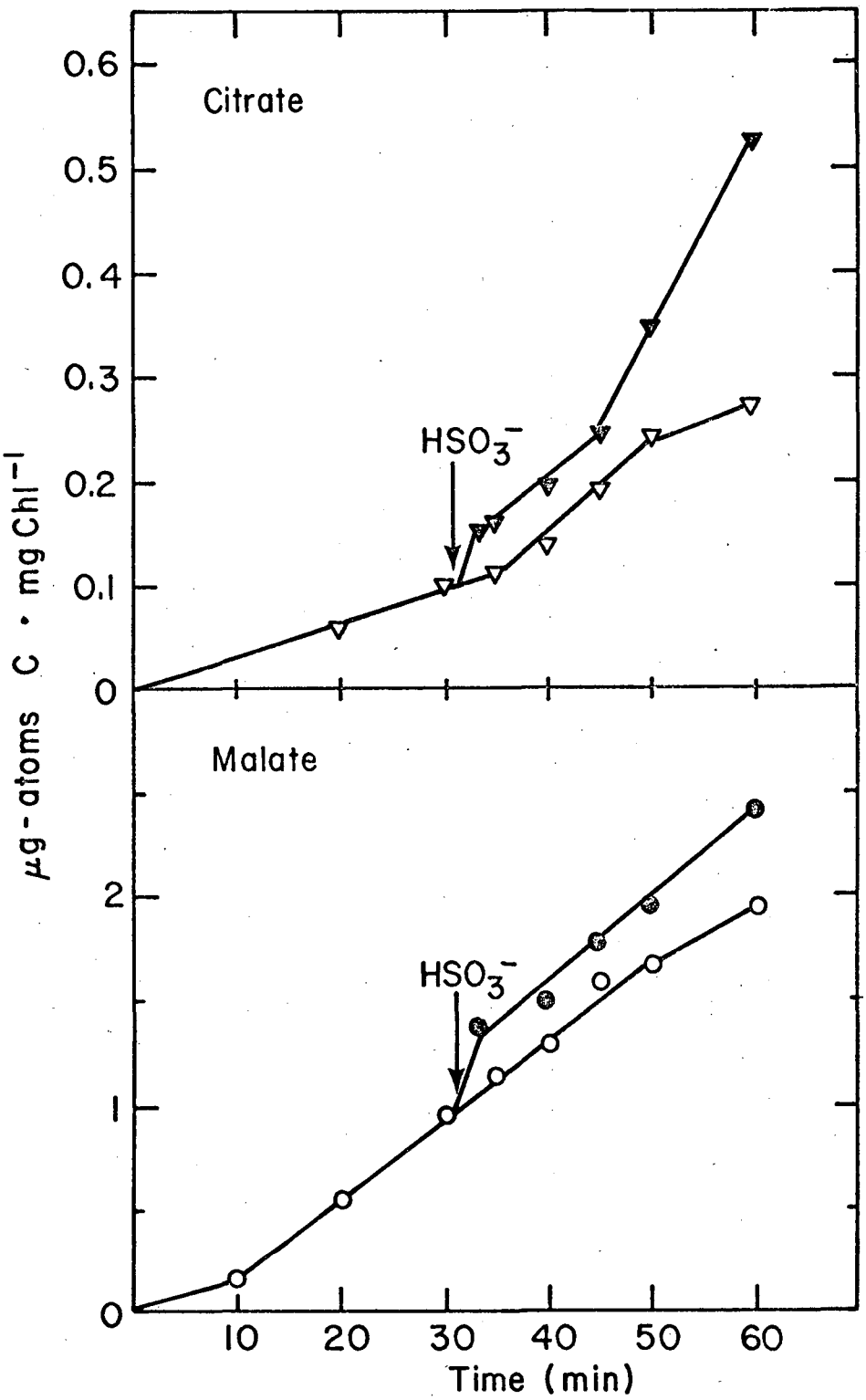

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Fig. 4

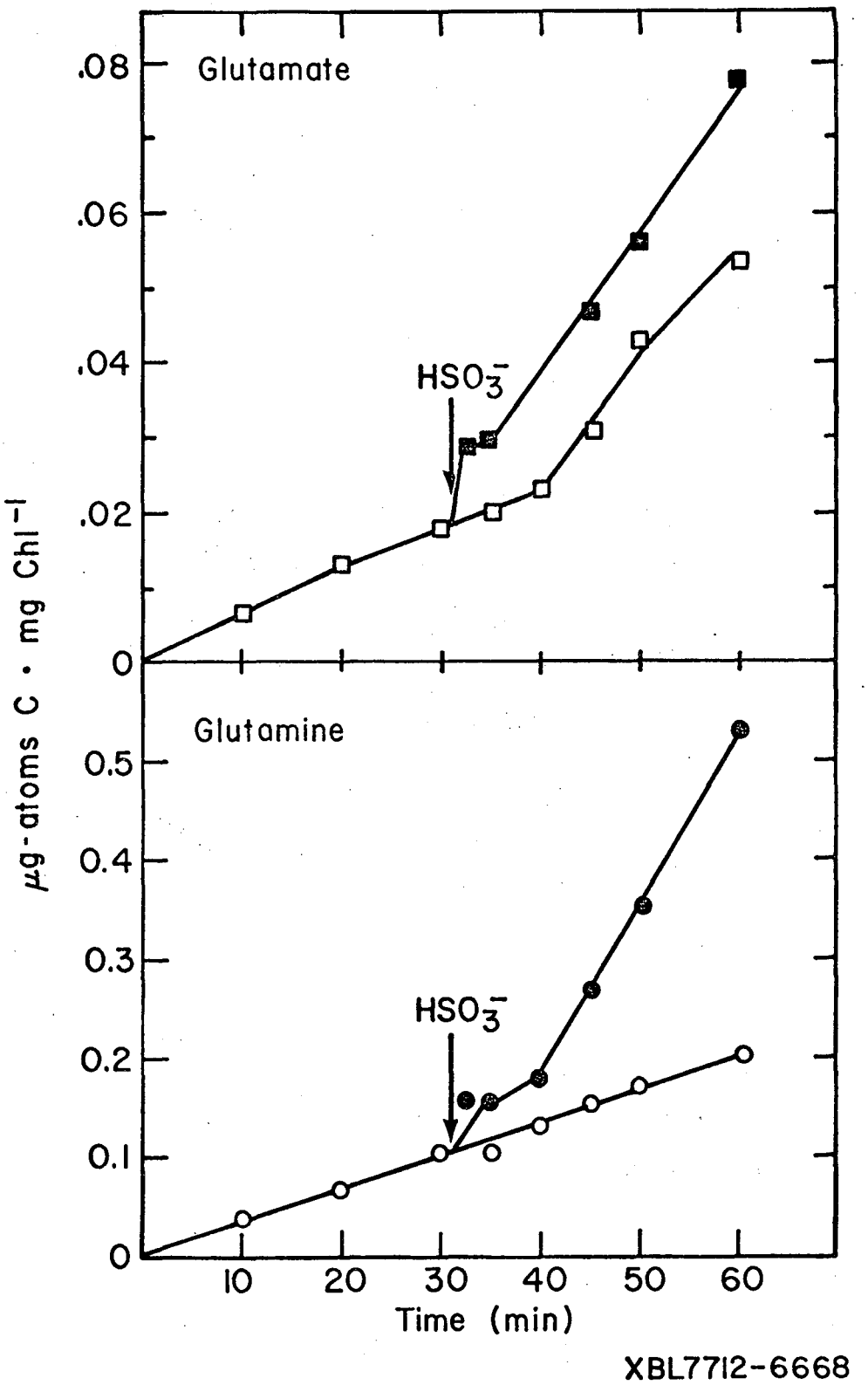

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Fig. 5

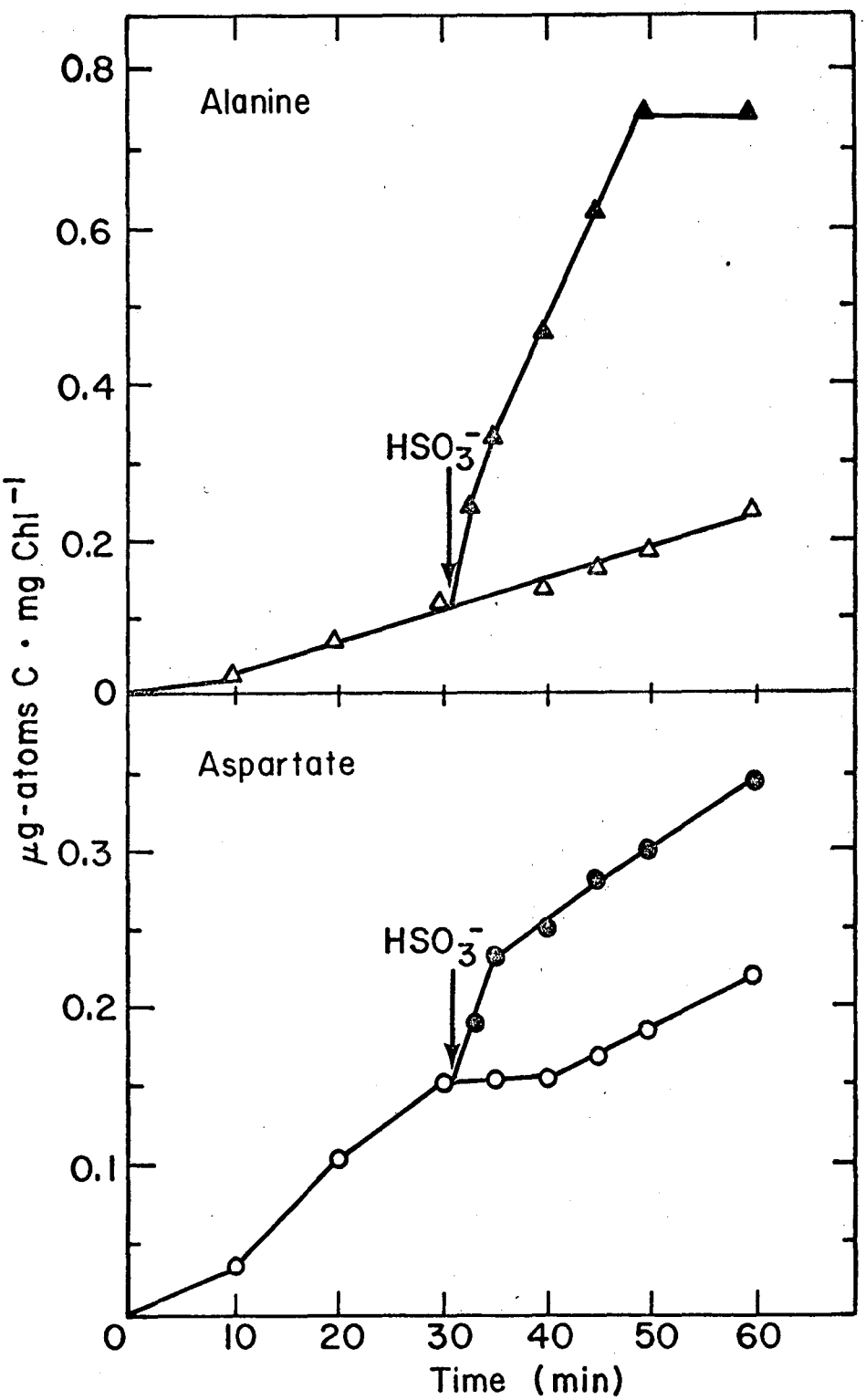

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Fig. 6

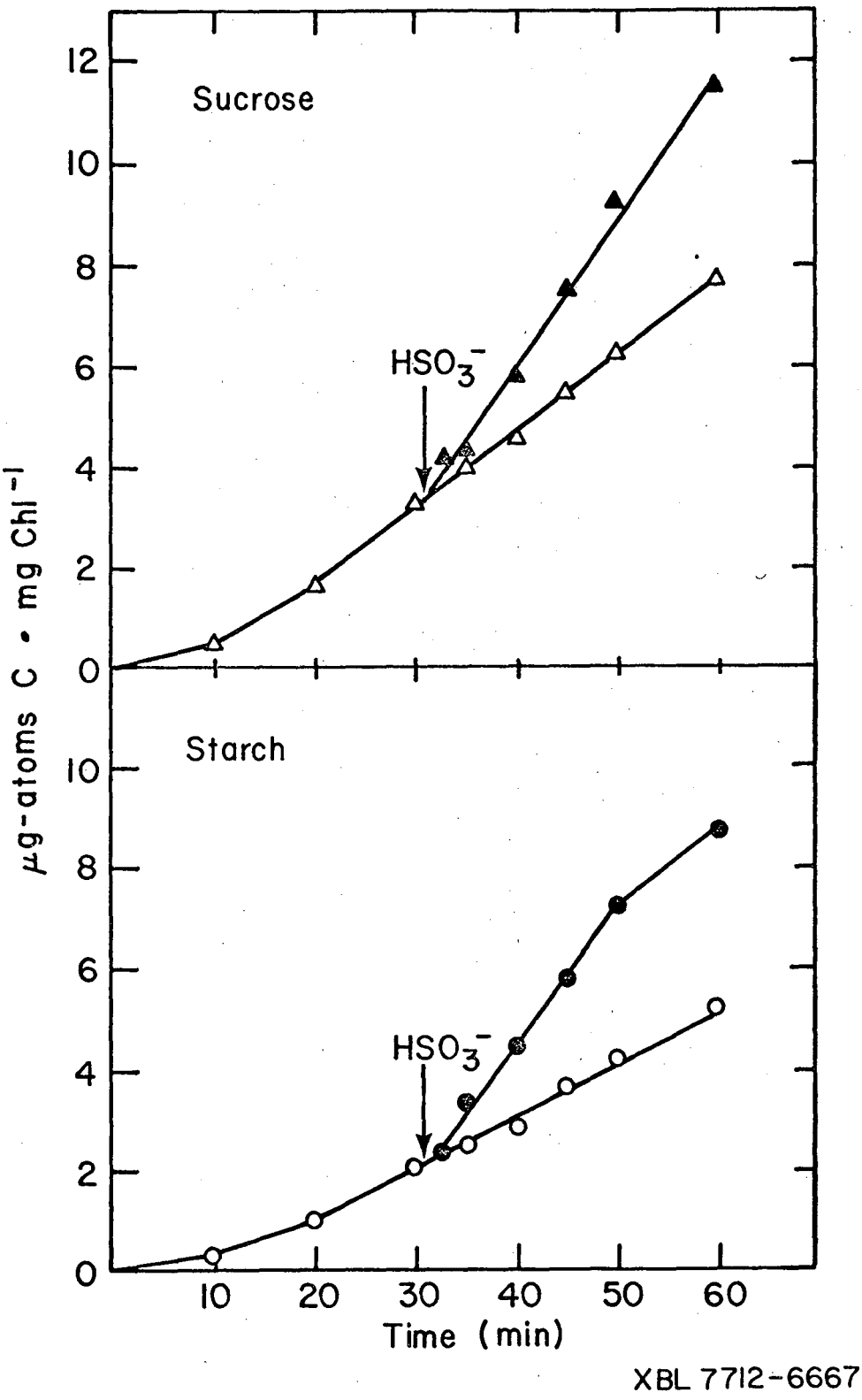

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Fig. 7

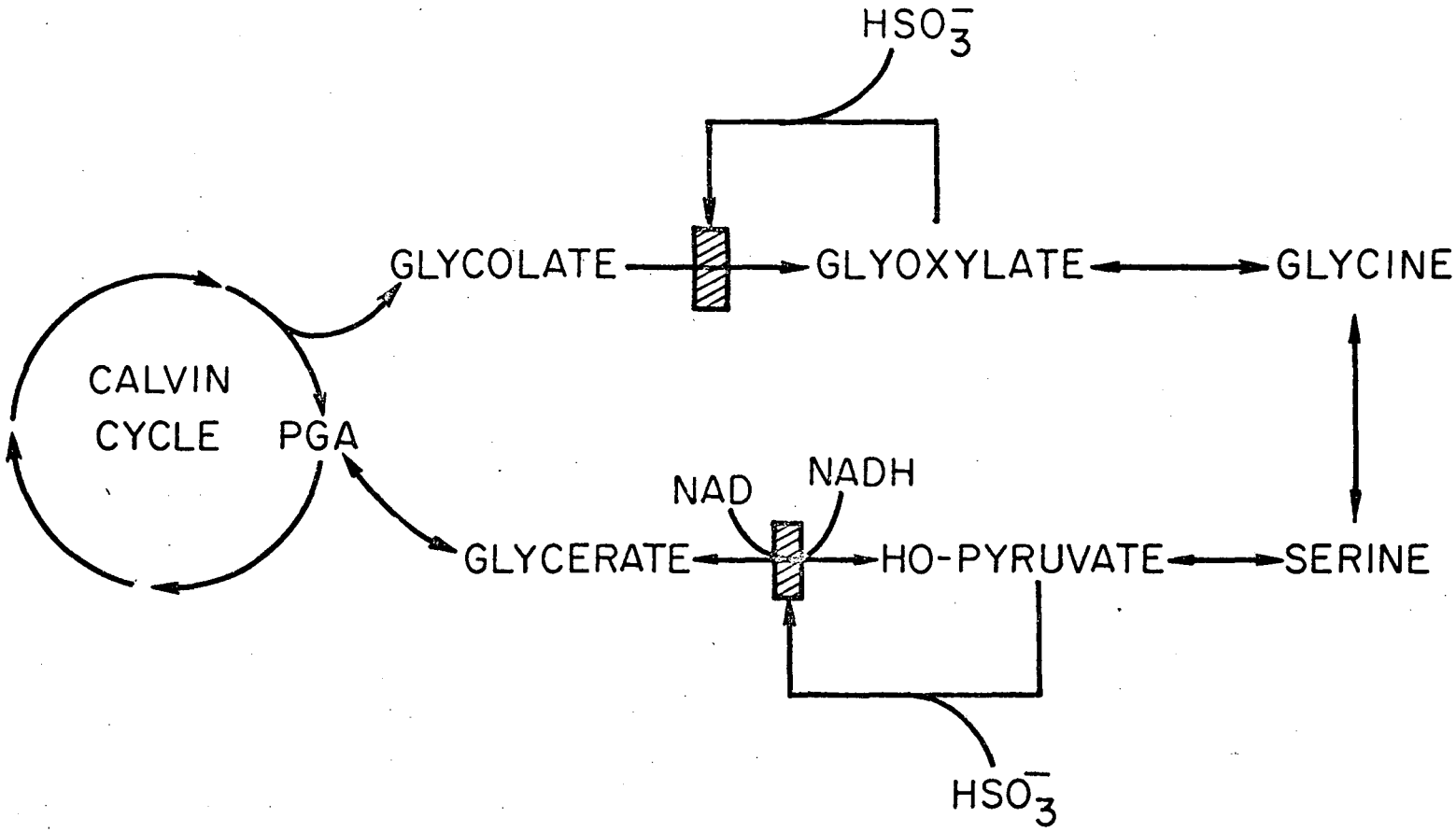

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Fig. 8

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