Effect of Light on Respiration and Development of Photosynthetic Cells

Renewal Application
and
Progress Report

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DISCLAIMER

ABSTRACT

The biophotolysis of water by photosynthetic cells resulting in the formation of hydrogen gas is of prime concern. That algal cells require both photosystems to complete this process is established. That a reduced carbon source can be photoxidized to release hydrogen and carbon dioxide has been proven. On the other hand, whether water is split to hydrogen and oxygen by the intact cell adapted to a hydrogen metabolism is an open question. A reconstituted preparation of higher plants can split water into its two components. A reconstituted algal preparation will be evaluated with respect to a similar reaction. If hydrogen and oxygen are produced in vitro, what then regulates the cell into controlling this reaction during the onset of a hydrogen metabolism.

The substrate for photorespiration is glycolic acid. The synthesis of this simple acid remain controversial. A new preparation of the spinach chloroplast has been developed which allows many compounds hitherto uncapable of crossing the organelle envelope to affect directly the carbon metabolism. We plan to use this preparation to evaluate the many proposed mechanisms of glycolate formation. Thus ribulose-1,5-diphosphate, hydroxypyruvate, hydroxypyruvate phosphate, oxaloacetate and fructose-6-phosphate will be incubated under varying conditions and glycolate yields will be monitored. Conditions such as pH, substrate concentration and oxygen partial pressure will be varied to determine accordance with in vivo conditions.

Progress Report

I. Hydrogen metabolism in the intact cell

A. Contribution of the photosystem

We have continued to elaborate on the effect of acetate on H_2-photoevolution by intact cells of Chlamydomonas reinhardi (wild type), Chlamydomonas reinhardi (F-60, a mutant lacking phosphoribulokinase) and Scenedesmus obliquus (wild type).

There is very little doubt that both photosystems contribute to H_2-photoevolution. This is demonstrated in Table I where with the C. reinhardi (wt) and S. obliquus, DCMU impairs H_2-photoevolution about 50%. A similar observation was made by Stuart and Gaffron (1, 2). Whether an organic donor functions in both photosystems or whether only in PS I with H_2O serving as donor in PS II is an unresolved question. Our research has been dedicated to this question.

It should also be noted in Table I that 3mM acetate stumulates H_2-photoproduction. This finding is a confirmation of an isolated observation of Healey (3). He concluded that acetate is oxidized via the citric acid cycle resulting in the evolution of CO2 and H_2. Our data establish that acetate is not an electron donor since acetate-^{14}C accumulated within these anaerobic cells at the rate of 0.1 µmol while H_2 was evolved at a rate of 25 to 35 µmol per mg chlorophyll per hour. Repetition of these experiments with acetate-^{12}C and in the presence of $^{14}CO_2$ revealed no significant $^{14}CO_2$ fixation. Thus, the lack of $^{14}CO_2$ evolved was not due to its refixation. We

would conclude that an anaerobically functioning citric acid cycle is not present in these H_2 evolving cells. The effect of acetate is elsewhere.

Since acetate was not photo-oxidized, clearly the acetate molecule per se was affecting H_2-photoevolution. Therefore, we determined the effect of acetate both on CO_2 and H_2 metabolism. It is well-known that accompanying H_2-photoevolution is CO_2 formation. This observation does not necessarily imply they derive from the same source. Most likely they do not since as shown previously (4), photohydrogen metabolism occurs in two phases: (a) a rapid one sensitive to DCMU and not necessarily coupled to CO_2 evolution and (b) a slow phase of H_2-photoevolution insensitive to DCMU and apparently coupled to CO_2 formation.

As seen in Figure 1, in the initial phase, H_2 dominates and little if any CO_2 is produced. With time, the H_2:CO_2 ratio approaches 2. In the dark, the ratio of H_2:CO_2/is equal to 1 (not shown). These data would be in accordance with our working hypothesis presented in Figure 2. In the light, the reoxidation of NADH by photosystem I would lead to the additional pair of hydrogens. In addition, these kinetic data are in accord with an initial flow of H_2 not necessarily coupled to CO_2 production. In the later periods, CO_2 reaches a value of 0.5 that of H_2. Clearly the ratio varies (see ref. 5) and would be expected if the CO_2 and H_2 derive from different sources.Also the ratio will reflect the growth conditions.

Interestingly, 3mM acetate while stimulating H_2-photoproduction (Table I, Figure 2) blocks CO_2 evolution. Thus acetate is not photo-oxidized by the citric acid cycle since the initial export of electrons would be isocitric acid dehydrogenase, an enzyme which catalyzes reduction of NAD coupled to CO_2 evolution (oxalosuccinic acid decarboxylase).

Therefore, it would appear that acetate is blocking the H_2-photoevolution coupled to CO_2 production or the metabolism of the organic donor has been blocked between PGA (glycerate-3-P) and pyruvate (Figure 2).

That acetate affects the DCMU-sensitive site (rapid H_2 not coupled to CO_2) rather than the DCMU-insensitive site is clearly demonstrated in Figure 3. While endogenous H_2-photoevolution is blocked only 10% by 10 μM DCMU, the acetate stimulated photoevolution is halted by 60-70%. Thus, there appear to be two separate pools of hydrogen donors. Since the DCMU-sensitive one leads to H_2 but not CO_2, the donor could be H_2O or an organic donor yielding electrons but not CO_2. An example would be the DCMU-sensitive oxidation of "active glycolaldehyde" to glycolic acid as demonstrated by Shain and Gibbs (6) or hydrogen peroxide (7). This section is concluded by noting that Stuart and Gaffron (1, 2) made a similar conclusion using the uncoupler, Cl-CCP which at high concentrations is an inhibitor of the H_2O-splitting act (8,9). This conclusion will be discussed in the Proposal.

B. Effect of acetate on modulating H_2-photoevolution

In the late and early 1960's, a considerable effort was devoted to carbon dioxide and the Hill reaction (10,11,12). The reversible phenomenon of dependence of the Hill reaction on CO_2 was demonstrated originally by incubating the chloroplast particles with KOH. Good (13) reported that small monofunctional anions such as chloride, formate and especially acetate in millimolar concentrations correlated with the loss of CO_2. In addition, the effects were

synergistic. West and Hill (14) observed that removal of CO_2 was hastened by these anions shortening incubation time over KOH from 2-3 hours to minutes. More recently, the Govindjee laboratory (15, 16, 17) reported that PS II but not PS I reactions were dependent upon catalytic amounts of CO_2.

Acetate like the more intensely studied ammonia can act as an uncoupler. This uncoupling effect in grana has been explained as a collapse of the proton gradient, caused as protons inside the thylakoid react with uncharged acetic acid which moves readily across the thylakoid membranes (18). Thus Heath and Leech (19) have shown that acetate blocks CO_2 fix in the isolated spinach chloroplast and this impairment can be removed by an equivalent level of ammonia.

Hitherto, the acetate effect on the Hill reaction has been solely with isolated chloroplasts. In preliminary experiments (Figure 4) benzoquine reduction assayed as O_2 evolution in intact cells of Chlamydomonas is sensitive to millimolar quantities of acetate. Formate will substitute for acetate (Fig. 5). The inhibition decreases at higher pH (data not shown) since the unionized acid is apparently the active species. Formate is presumably less inhibitory since its pK is considerably less than acetate. Thus, acetate may well block PS II by removing CO_2. Acetate has a lesser effect on H_2-photoevolution (not shown).

C. Effect of starvation on H_2-photoevolution

The endogenous rate of H_2-evolution falls off with starvation but evolution is restored by the addition of acetate. It would appear that the slow phase (CO_2 and H_2) derived from fermentation and insensitive to DCMU is eliminated by starvation but that the PS II catalyzed system

is accelerated by acetate. Therefore, in order to determine whether water or an organic donor is photooxidized via PS II, various parameters were followed during starvation: (a) respiration (RESP); (b) photorespiration (PR); (c) photosynthesis (PS), (d) H_2-photoevolution by whole cell (LH_2-WC) and (e) H_2-photoevolution by Scenedesmus particles fortified with ferredoxin, hydrogenase and NADH (LH_2-BP). The latter reconstituted system was reported by Amotz and Gibbs (20) demonstrating that NADH can donate to a "quinone" resulting in a DCMU-insensitive mode of H_2 evolution. The data are recorded in Figure 6.

The first activity to decline was endogenous H_2 evolution in whole cells; activity virtually ceased in 48 hours. During this time, photoreduction (PR)which involves CO_2 reduction by H_2 in the presence of 10 μM DCMU remained at 100%, indicating no loss in hydrogenase activity. The further depletion of substrates with time is seen in the decline of aerobic respiration to the point of cessation after 100 hours. Longer periods of starvation resulted in a gradual loss of photosynthetic CO_2 fixation though not of the photosynthetic carbon reduction cycle. After 60 hours of starvation, the cell was capable of reducing added p-benzoquinone indicating an intact H_2O-splitting act. A gradual loss in the capacity of the chloroplast particles to evolve H_2 from NADH was observed after 100 hours.

At first glance of these findings, one could conclude that H_2O is not a donor since H_2-photoevolution ceases when the endogenous stores are released. On the other hand, the capacity of the photosystems to split water functions as monitored by photosynthesis and

the release of O_2 coupled to p-benzoquinone reduction. In sharp contrast, addition of acetate reveals H_2-photoevolution even though endogenous H_2 formation has ceased. Is H_2 derived from H_2O or from an organic donor which is mobilized by acetate releasing electrons but no CO_2. Clearly, the organic donors funneling electrons into the two photosystems would have to differ. If there is a common donor which seems unlikely, then acetate would alter its metabolism.

Bishop et al. (4) has reported mutants of Scenedesmus which do not photosynthesize, release no hydrogen, can photoreduce and can reduce dichlorophenolindophenol with diphenylcarbazide as donor. He concluded that H_2 is not produced since the H_2O-splitting act is impaired. On the other hand, the diphenylcarbazide input in PS II functions. Would acetate reveal H_2-evolution in this cell. Unfortunately, this was not tested.

Another factor complicating the starvation approach is that recently reported by Kulandaivelu and Senger (21, 22). According to these authors, PS II and PS I activities decline in aging cultures of Scenedesmus . The activity declining most rapidly was quinone photo-oxidation. This reaction may well be present in our starved cultures and account for some of the data. However, in our preparations NADH $\rightarrow$ H_2 decreased only slowly. Since this activity is sensitive to quinone analogs, it would seem that the rate-limiting step is elsewhere.

II. Photorespiration

A. The Warburg Effect

The inhibition of photosynthesis by O_2 has been termed the Warburg effect. The effect is reversible and appears related to glycolate formation, the substrate of photorespiration.

The effect of O_2 has been attributed to:

(1) inhibition of -SH containing enzymes(23, 24).

(2) the drainage of carbon in the form of glycolate from the photosynthetic carbon reduction cycle resulting in a limitation of ribulose-1,5-diphosphate (25, 26).

Now we report that H_2O_2 produced by broken chloroplasts in an "intact plastid" preparation is a causal agent of the oxygen mediated inhibition of photosynthesis.

We have continued our interest in peroxide since we proposed a number of years back that H_2O_2 or perhaps the superoxide anion radical was involved in glycolate production (6). We also showed that ascorbate had a striking enhancement on glycolate formation (27).

The Warburg effect is more pronounced at the higher pH. Also that catalase protects to some extent the O_2 inhibition. Since catalase does not cross the envelope of the intact plastid, catalase protects CO_2 fixation by removing peroxide photogenerated by the broken chloroplasts. The peroxide is presumably formed by the reoxidation of reduced ferredoxin by O_2.

Ascorbate and ribose-5-P, an intermediate of the photosynthetic carbon reduction cycle, protect against externally

added H_2O_2. Since peroxide, ribose -5-P and ascorbate penetrate the chloroplast, the latter protects apparently against internally generated peroxide.

B. Chloroplast preparation permeable to large molecules

Hitherto two kinds of isolated chloroplast preparations have been available to monitor CO_2 fixation: (1) intact (2) reconstituted, a combination of stromal enzymes and lamellar membranes (28). Each has its purpose. Unfortunately, preparation 1 is not permeable to hexose phosphates, pyridine nucleotides, nucleotide triphosphates and so forth while the latter one possesses few controls and has other disadvantages.

We have prepared an intermediate preparation which retains the membrane albeit a very leaky one. It is prepared in the following way. Deveined spinach leaf was diced and 12-15 g of this tissue was homogenized in a Virtis blender (50% line voltage) with 50-60 ml of grinding medium for 5 sec. The grinding medium contained 0.05 M HEPES (pH 6.8), diNaEDTA (5mM), 1mM $MgCl_2$, 1mM $MnCl_2$, 1mM $Na_4P_2O_7$, 0.33 M Sorbitol. The homogenate was filtered through two layers of mira-cloth. The resulting filtrate was centrifuged at 455 g for 50 sec. The pellet obtained was then washed with 50ml wash medium, containing 0.33M Sorbitol, 0.04M HEPES at pH 7.5. The suspension was then centrifuged at 755g. The pellet was then resuspended in 10 ml of breakage medium containing 0.05M TRICINE (pH 8.5), 1mM EDTA and varying concentration of $MgCl_2$. The suspension was allowed to sit in the cold (4°c, in an ice bucket) for 10 minutes, with shaking occasionally. The suspension was then centrifuged at 10,000g for 10 minutes. The pellet obtained was then resuspended in the same breakage medium in a final volume of 4 ml. The suspension is then called the $MgCl_2$ treated particles.

Table II shows that some but not all enzymes are retained "within" the envelope as the Mg^{2+} concentration is increased. In contrast to "intact" plastids prepared in 0.3M sorbitol, these particles are penetrated by ferricyanide. The rate of CO_2 fixation by this particle is roughly 3 µmol per mg chlorophyll per hour. In the absence of Mg^{2+}, the rate is about 1.

The remaining figures indicate the usefulness of this preparation in studying regulation at a level more organized than the reconstituted system of stroma and grana.

Figure 7. The preparation is ferredoxin dependent. Clearly some ferredoxin has been retained but the enzymic apparatus is sufficiently exposed to be dependent upon the iron-sulfur protein.

Figure 8. In contrast to ferredoxin, increasing NADP concentration initially stimulates CO_2 fixation but, in turn, inhibits. In order for the Calvin cycle to function, fructose-6-P and triose-P are constantly required. Glycerate-3-P is the primer from which the ketohexose-P and triose-P are derived. Clearly increasing NADP in an unknown mode blocks their formation.

Figure 9. NAD, in contrast to NADP, stimulates slightly but does not inhibit CO_2 fixation. Apparently the NAD-triose-P dehydrogenase can function to a limited extent.

Figure 10. That fructose-6-P is required for CO_2 fixation is illustrated here. Surprisingly, glucose-1,-P and glucose-6-P which are in equilibrium with fructose-6-P do not mimic this compound.

Figure 11. Gluconate-6-P which is known to block ribulose-1,5-diP carboxylase is an inhibitor. The concentration is far higher than that required for the isolated enzyme.

Figure 12. As expected ADP stimulates but unexpectedly inhibits at higher concentration. This preparation has a high concentration of myokinase (ADP + ADP → ATP + AMP). Thus, ATP and/or AMP may well be blocking ribulose-5-P kinase.

Figure Legends

Figure 1. The evolution of CO_2 and H_2 by Chlamydomonas reinhardi F-60 in the presence and absence of 3 M acetate.

Evolved gases were differentially trapped (± alkaline pyrogallol). Light intensity (100 w/m^2) did not produce measurable O_2.

Figure 2. The coupling of electron transport to H_2 and CO_2 photoevolution in algae.

The donation of electrons for H_2-photoevolution to probable sites in electron transport was elucidated by means of donors and inhibitors.

Figure 3. The effects of acetate and DCMU on the photoevolution of H_2 in F-60.

The presence of 10 μM DCMU inhibited the endogenous evolution about 20% but acetate-stimulated H_2-photoevolution by roughly 90%.

Figure 4. The inhibition of O_2 evolution by acetate in adapted cells of C. reinhardii.

The electron acceptor was benzoquinone.

Figure 5. Comparison of inhibition of Hill reaction by chloride, formate and acetate.

The electron acceptor was benzoquinone.

Figure 6. Effect of starvation of various photosynthetic functions of Scenedesmus obliquus.

Figure 7. Dependency of CO_2 fixation upon ferredoxin.

Figure 8. Dependence of CO_2 fixation upon NADP

Figure 9. Dependency of CO_2 fixation upon NAD

Figure 10. Dependency of CO_2 fixation upon fructose-6-P

Figure 11. Inhibition of CO_2 fixation by gluconate-6-P

Figure 12. Sensitivity of CO_2 fixation to ADP

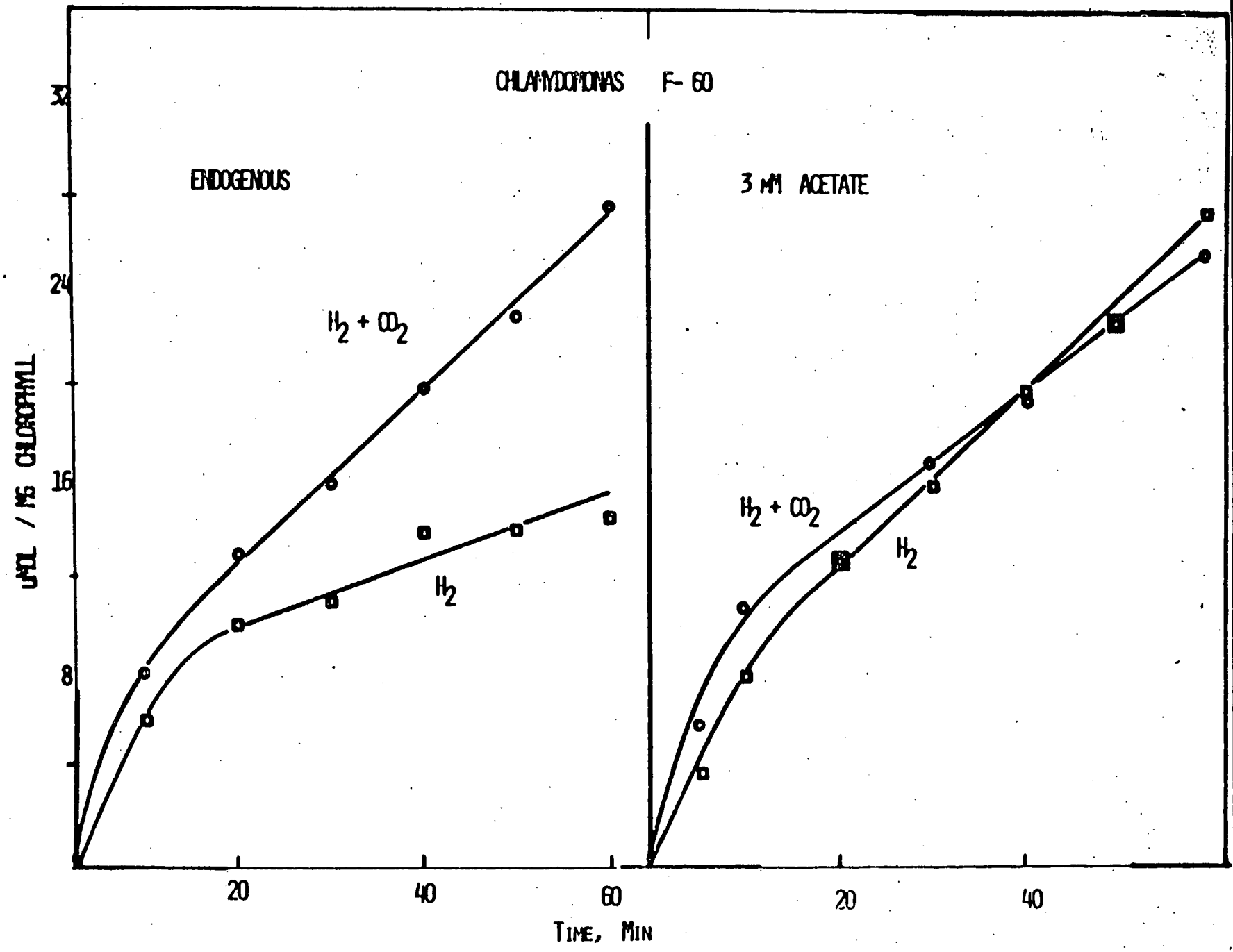

CHLAMYDOMONAS F-60
ENDOGENOUS
3 MM ACETATE
$H_2 + CO_2$
H_2
$H_2 + CO_2$
H_2
UMOL / MG CHLOROPHYLL
32
24
16
8
20
40
60
20
40
TIME, MIN

FIGURE 1

PHOTOEVOLUTION OF HYDROGEN

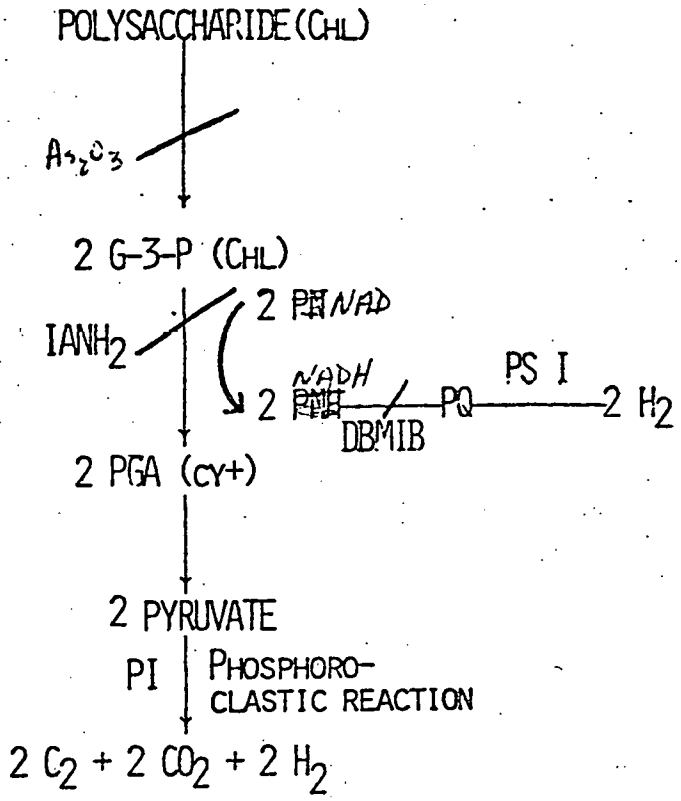


FIGURE 2

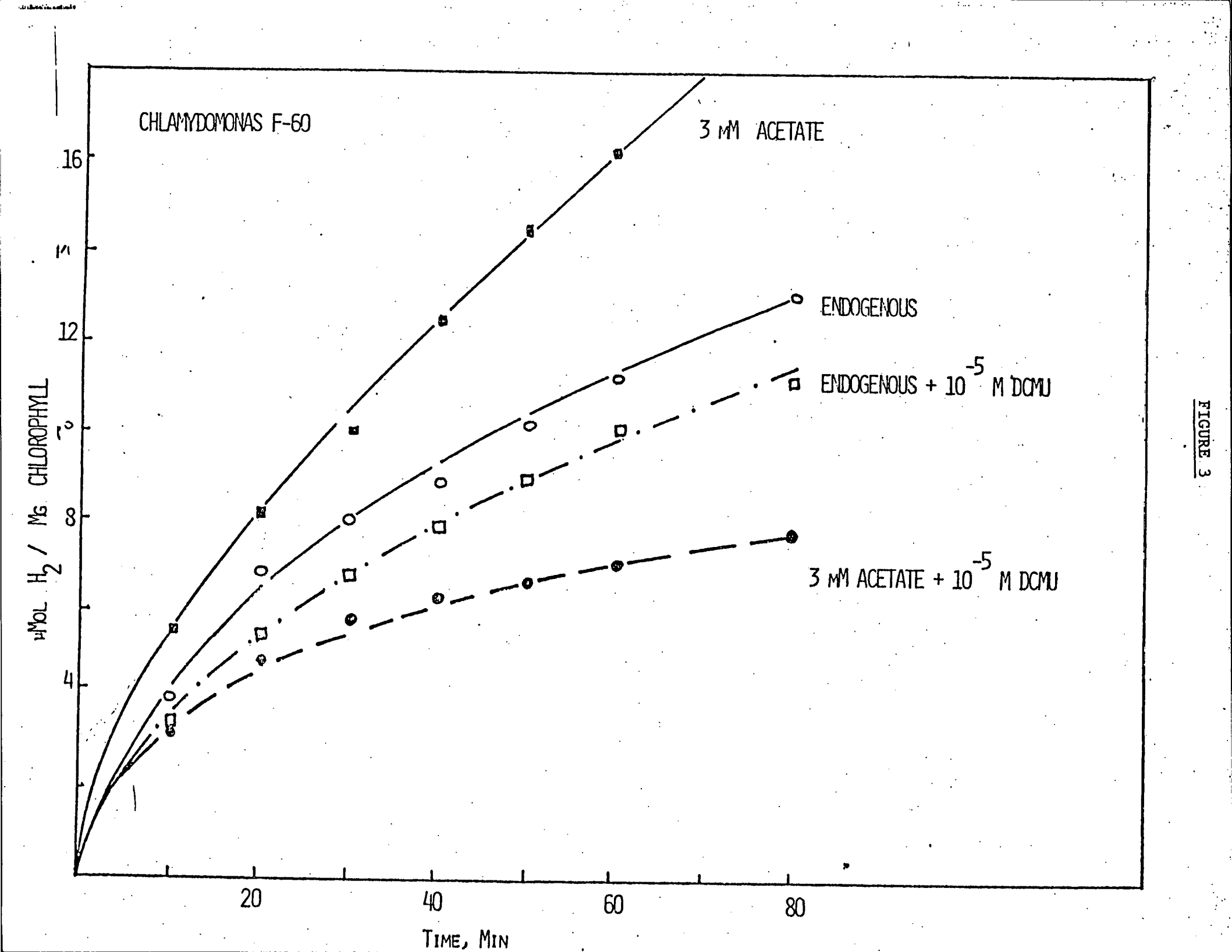
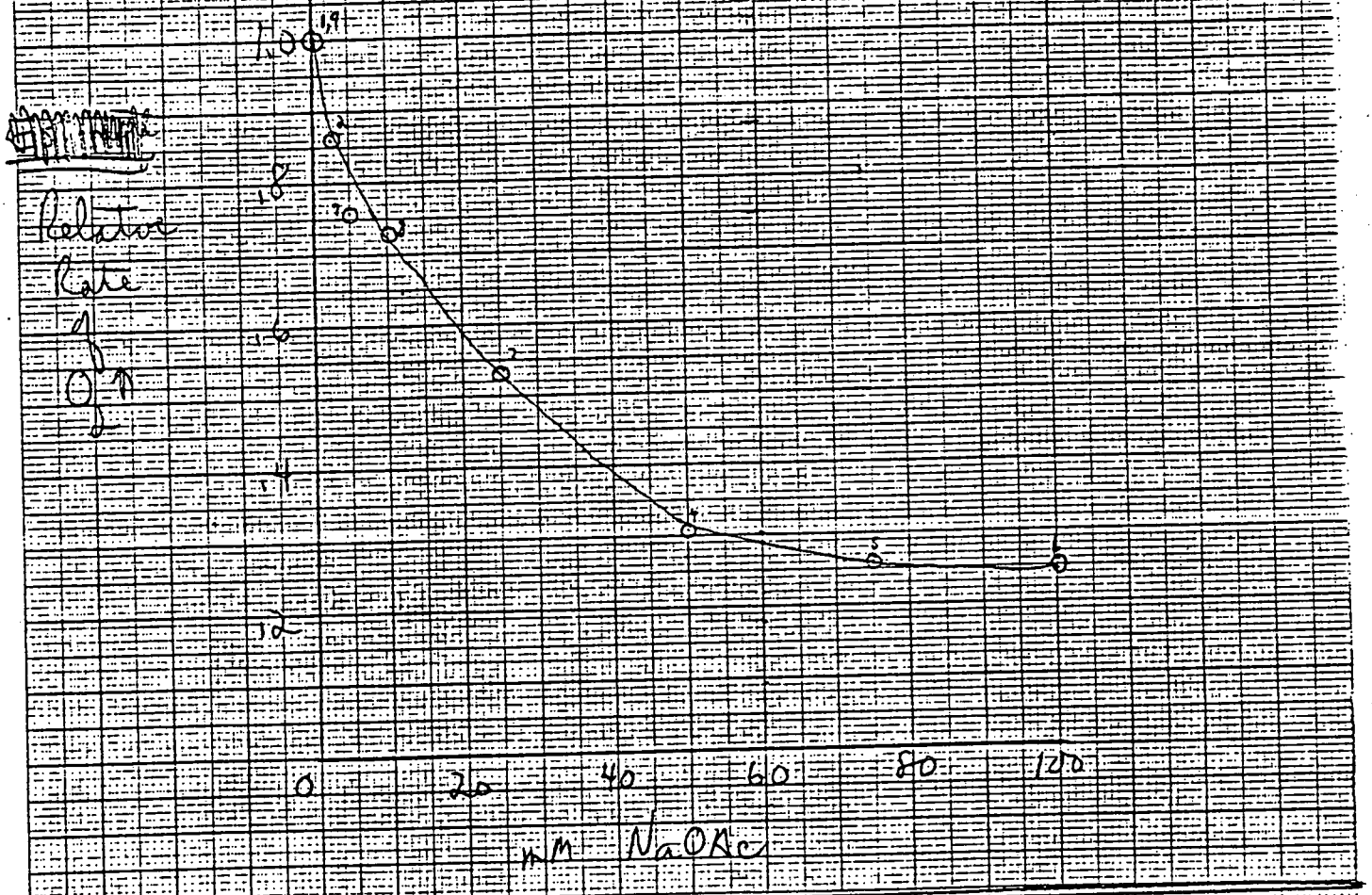

CHLAMYDOMONAS F-60
3 mM ACETATE
ENDOGENOUS
ENDOGENOUS + 10⁻⁵ M DCMU
3 mM ACETATE + 10⁻⁵ M DCMU
μMOL H2 / MG CHLOROPHYLL
TIME, MIN
4
8
12
16
20
40
60
80

FIGURE 3

H_2O → 6 ϕ / FeCN

pH 6.6 / Anaerobic / adapted cells (Creatine) / N_2



	(μmoles O_2 / mg chl / hr)	
Prey	Dark Respiration	O_2
cells	-34	+11
cell + FeCN/BQ	-3	+123

FIGURE 4

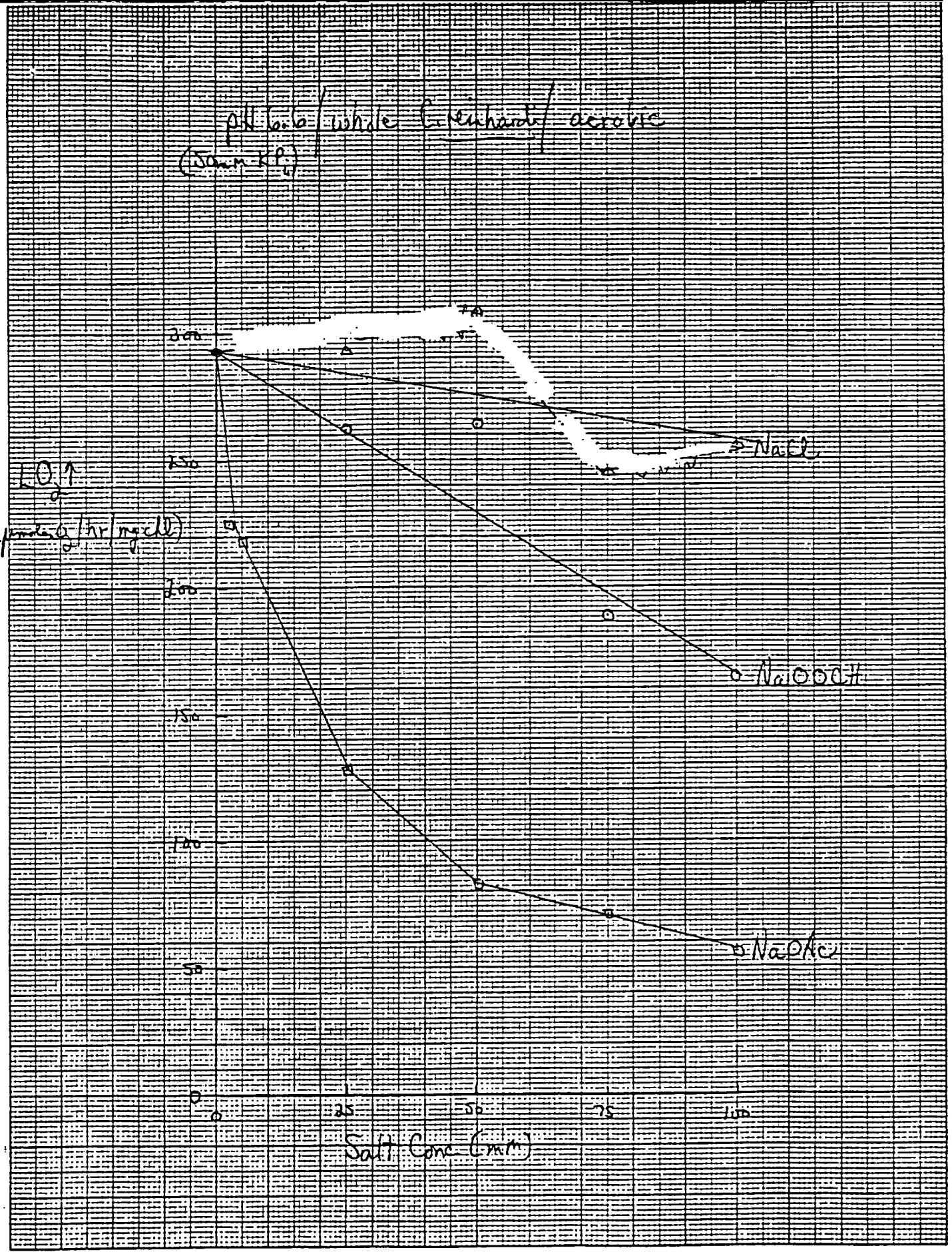


FIGURE 5

FIGURE 6

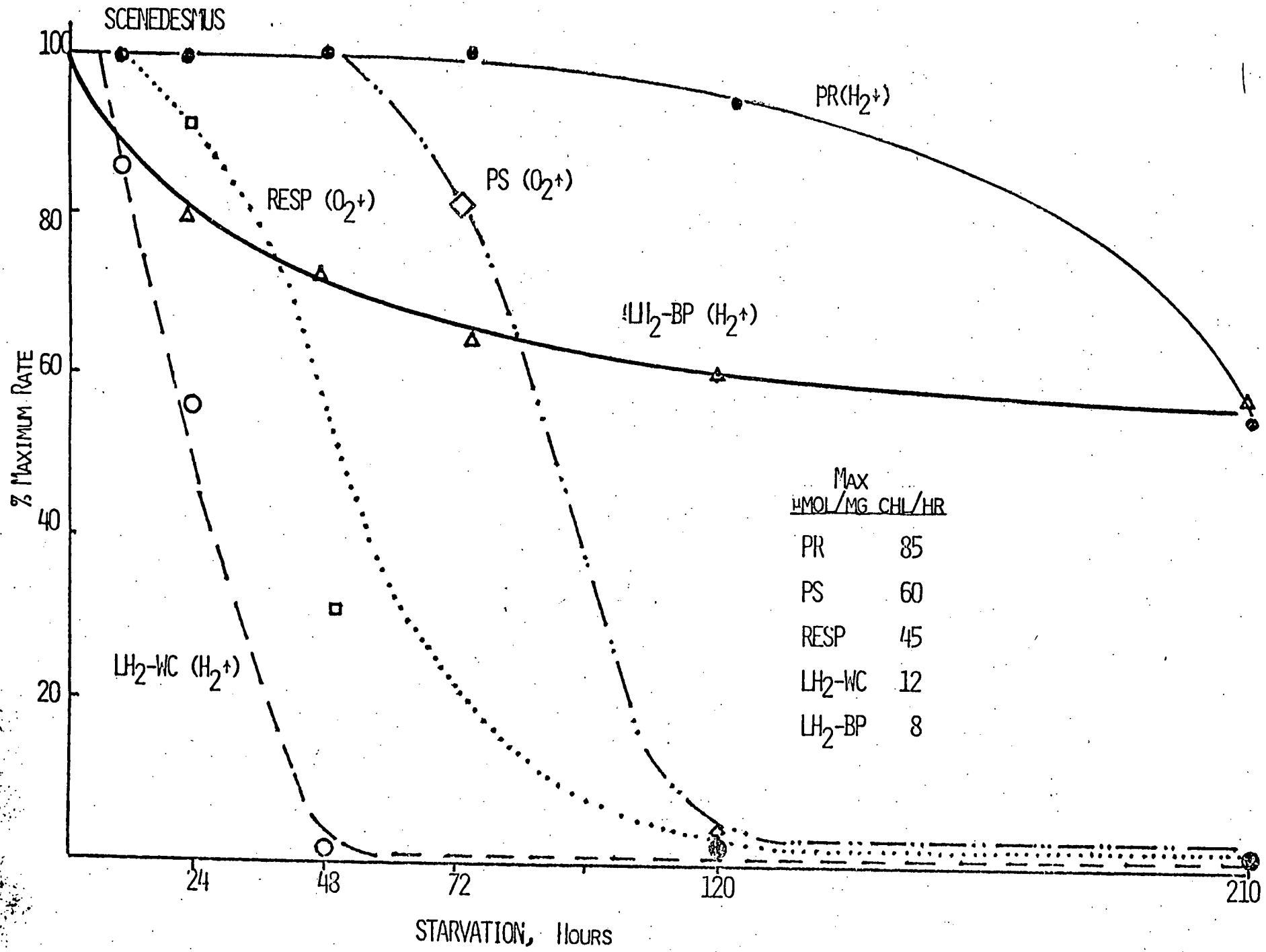

SCENEDESMUS
100
80
60
40
20
% MAXIMUM RATE
PR(H_2^+)
PS (O_2^+)
RESP (O_2^+)
LH$_2$-BP (H_2^+)
LH$_2$-WC (H_2^+)
MAX
µMOL/MG CHL/HR
PR 85
PS 60
RESP 45
LH$_2$-WC 12
LH$_2$-BP 8
24
48
72
120
210
STARVATION, HOURS

FIGURE 7

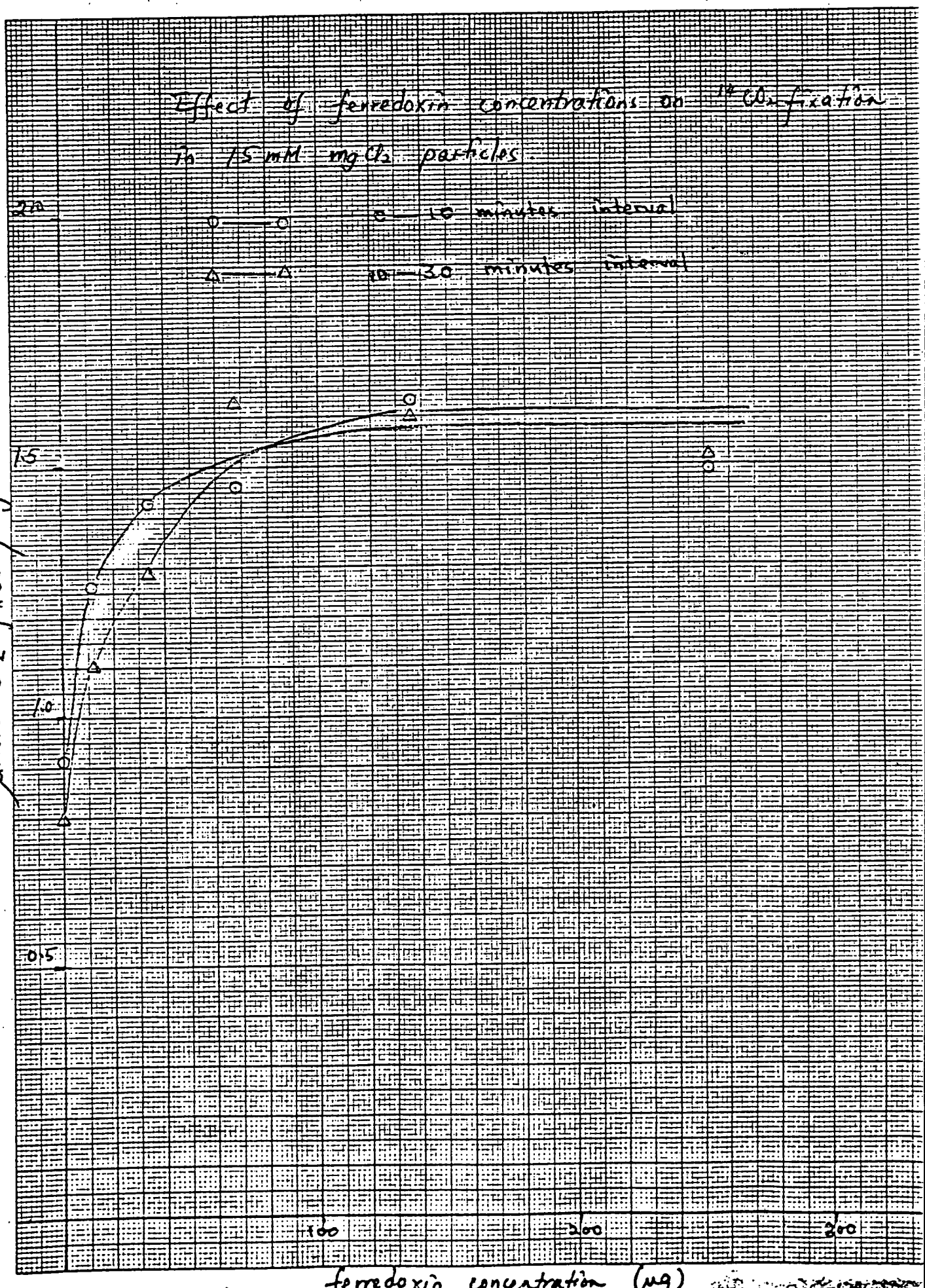


FIGURE 8

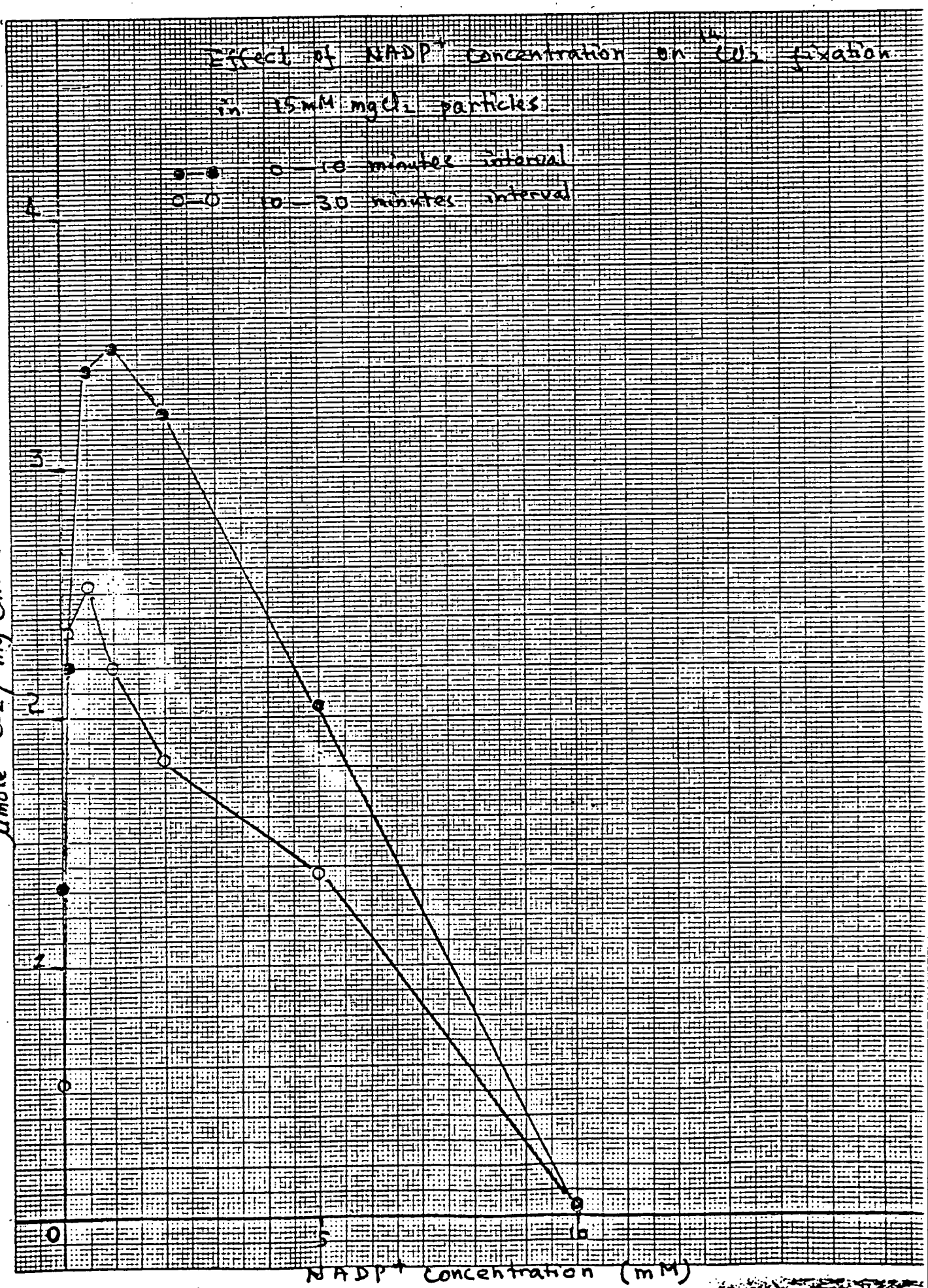


FIGURE 9

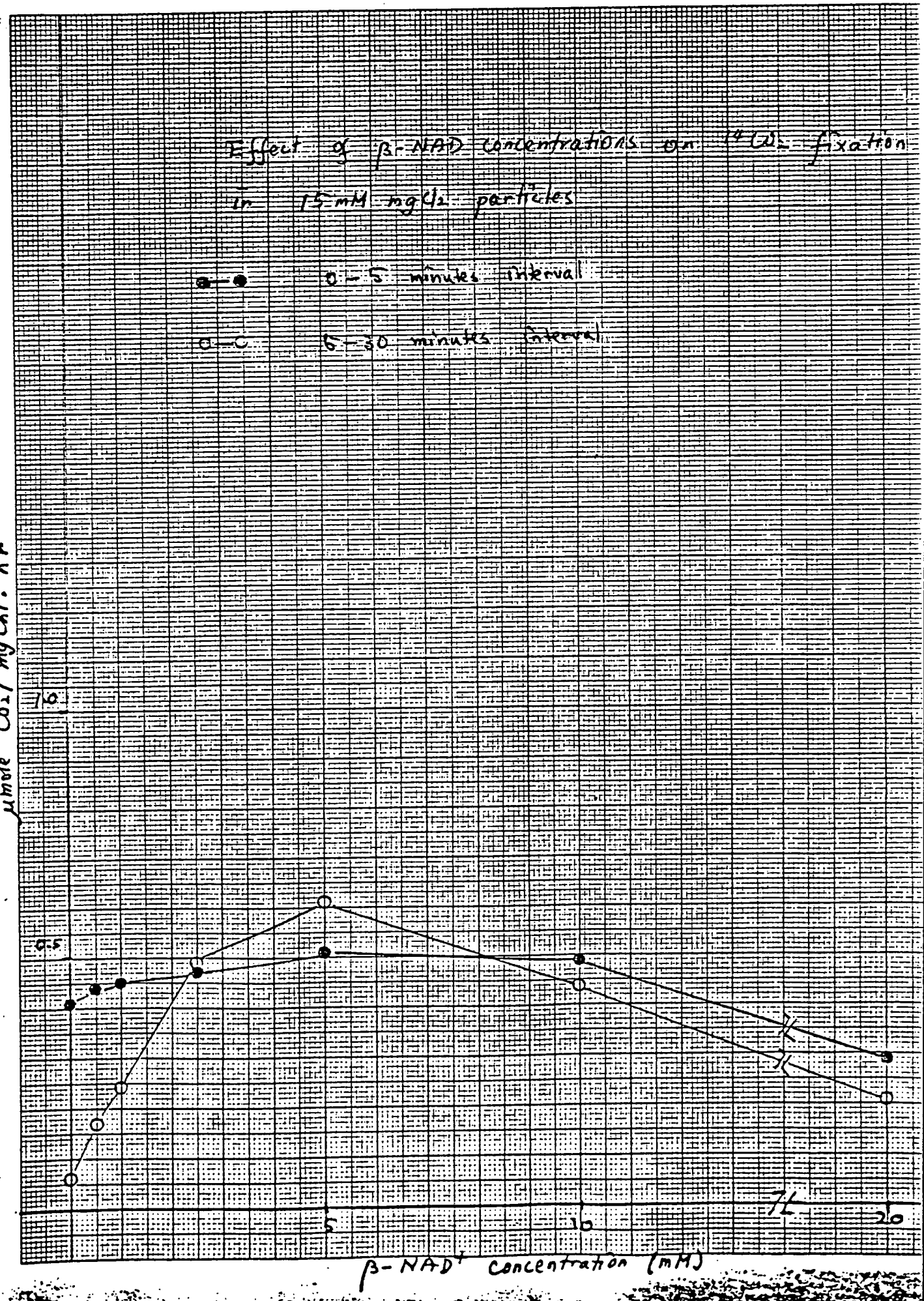

Effect of β-NAD concentrations on $^{14}CO_2$ fixation
in 15 mM $MgCl_2$ particles
0–5 minutes interval
6–30 minutes interval
μmole CO_2/mg chl. hr
16
0.5
1
5
10
20
β-NAD+ Concentration (mM)
461510
K&E 10 X 10 TO THE CENTIMETER 18 X 25 CM.
KEUFFEL & ESSER CO. MADE IN U.S.A.

FIGURE 10

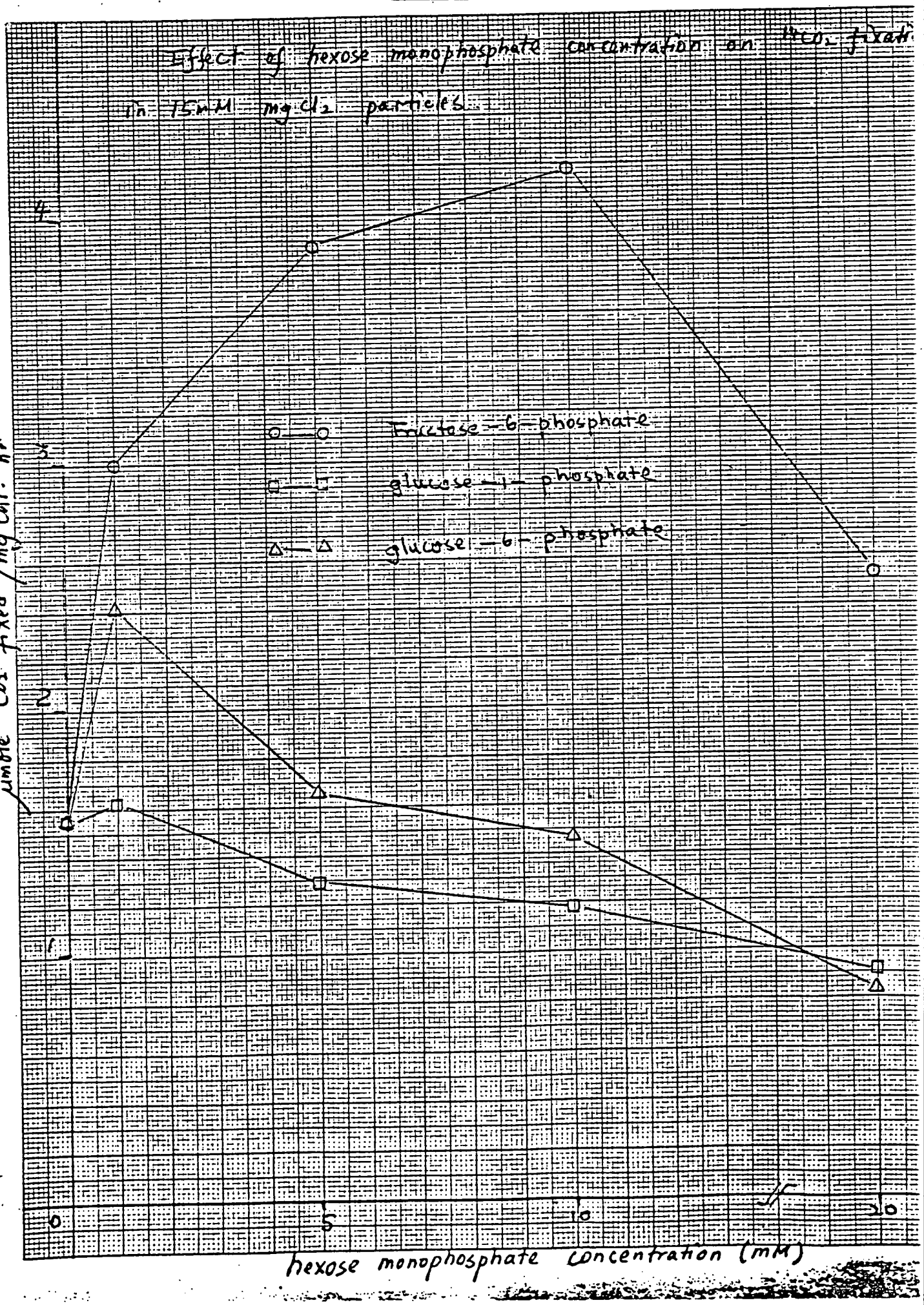
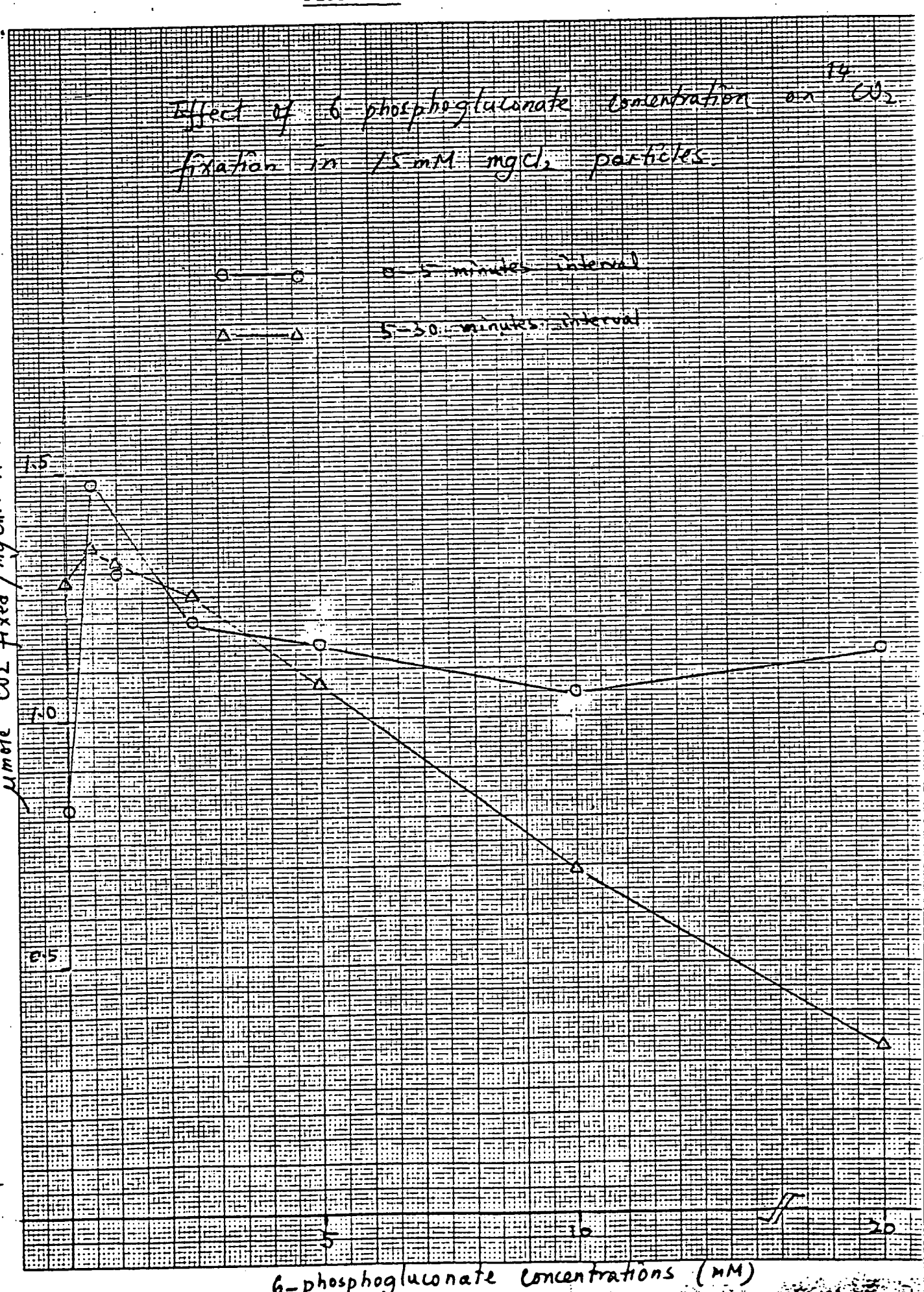


FIGURE 11



461510

KE 10 X 10 TO THE CENTIMETER 18 X 25 CM.
KEUFFEL & ESSER CO. MADE IN U.S.A.

FIGURE 12

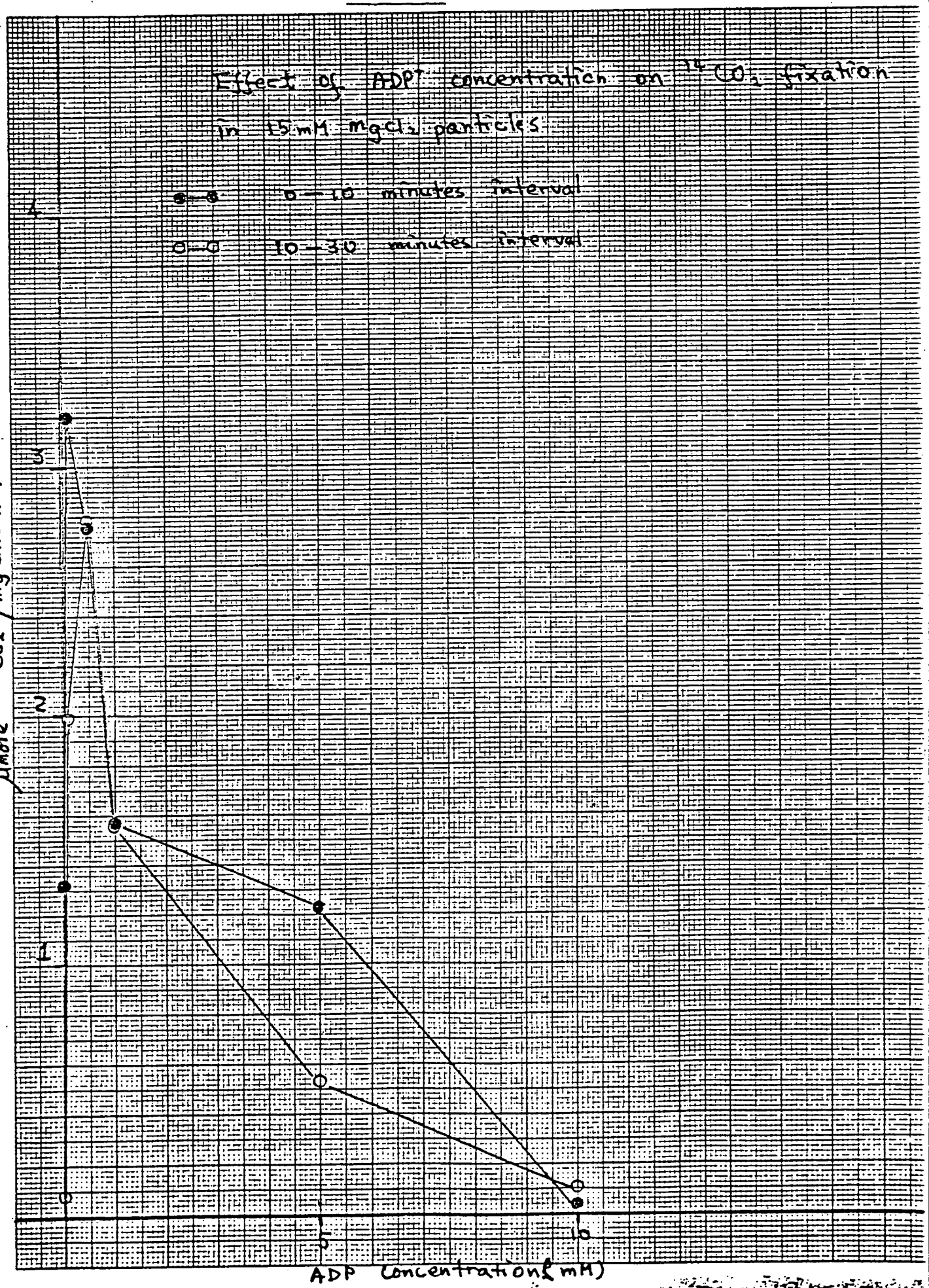

Effect of ADP concentration on $^{14}CO_2$ fixation
in 15 mM $MgCl_2$ particles
0—0 0 — 10 minutes interval
o—o 10 — 30 minutes interval
μmole $^{14}CO_2$ / mg Chl hr.
ADP Concentration (mM)
0
5
10
461510
K&E 10 X 10 TO THE CENTIMETER 18 X 25 CM.
KEUFFEL & ESSER CO. MADE IN U.S.A.

Table I

Effect of growth condition and acetate on hydrogen evolution

Organism	Endogenous μmol H_2/mg Chl·hr	+1ml Acetate μmol H_2/mg Chl·hr
C. reinhardi, wt		
Autotrophic	6	12
Photoheterotrophic	18	18
Photoheterotrophic + DCMU	9	8
Starved	1	5
C. reinhardi, F-60		
Photoheterotrophic	18	35
Photoheterotrophic + DCMU	10	10
Starved	3	8
Scendesmus obliquus		
Autotrophic	3	10
Photoheterotrophic	12	33
Photoheterotrophic + DCMU	6	15
Starved	0-1	8

Table II

Enzyme activities in different $mgCl_2$ concentration prepared particles

Enzymes/Activity*	mM $MgCl_2$ 0	10	30	50
PGA kinase	220	228	259	287
Triose-P isomerase	290	339	450	495
FDP aldolase	26	32	38	44
Phosphoglucomutase	4.4	9.8	13.9	15.3
FDPase, pH 8.5	10.5	19.6	17.8	23.1
Phosphoriboisomerase	25.8	125.0	-	-
Xylulose 5-P isomerase		25.8	-	-

*activity=μmole substrates consumed/mg Chl·hr