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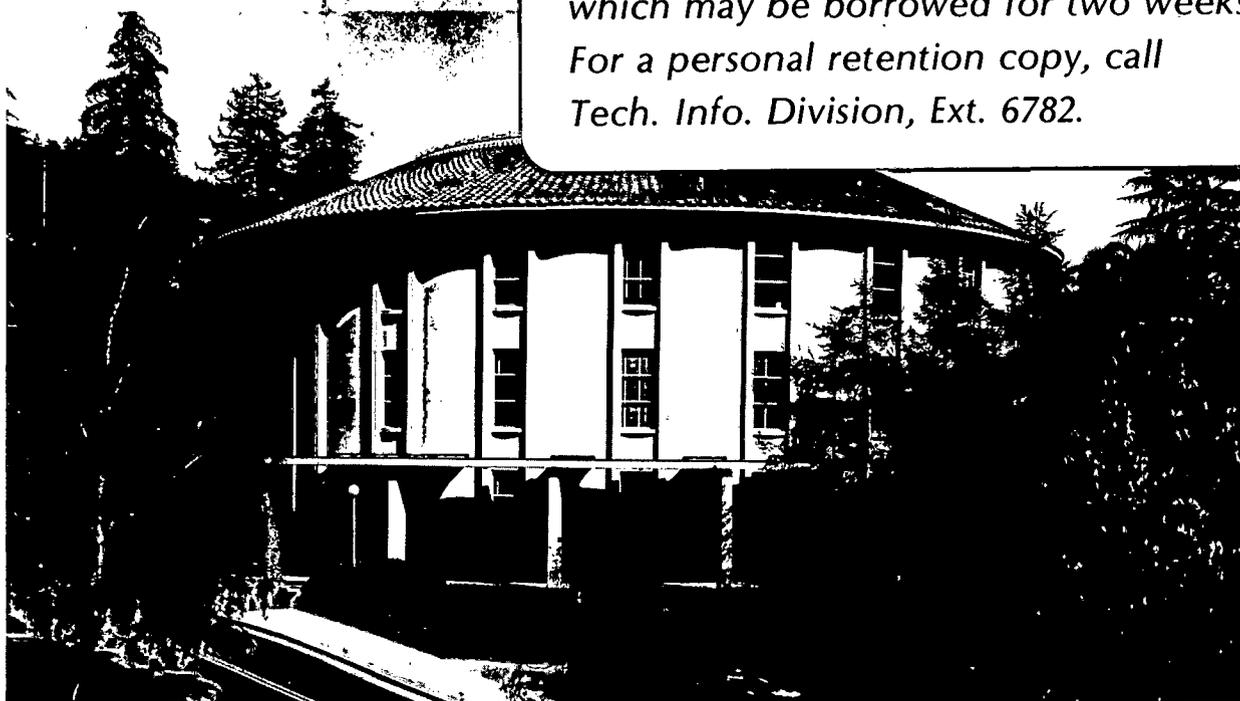
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COMPARATIVE EFFECTS OF METHYLAMINE

Tamotsu Kanazawa, Mark Distefano, and
James A. Bassham

February 1983

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AMMONIA REGULATION OF INTERMEDIARY METABOLISM
IN PHOTOSYNTHESIZING AND RESPIRING Chlorella pyrenoidosa:
COMPARATIVE EFFECTS OF METHYLAMINE

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ABSTRACT

Methylamine added to the medium of the unicellular alga Chlorella pyrenoidosa causes most of the shifts in carbon metabolism previously observed with ammonia addition. The most prominent and reproducible effect seen is a greatly increased flow of carbon from triose phosphates into alanine, aspartate, and glutamate, and acids of the tricarboxylic acid cycle including malate, fumarate, and citrate.

The large increase in synthesis of glutamine seen with ammonia addition does not occur with methylamine addition. It is concluded that accelerated glutamine synthesis per se is not the primary cause of the other regulatory shifts in carbon metabolism seen with ammonia (and now with methylamine) as has been proposed earlier.

Stimulation of phosphoenolpyruvate carboxylase by methylamine supports a previous proposal that the activity of this enzyme increases with a small rise in cytoplasmic pH resulting from entry of ammonia into the cytoplasm. Increases in the activity of pyruvate kinase and the rate of oxidation of triose phosphate remain unexplained. If these changes are due to change in ATP/ADP ratio, the cause for the change in this ratio must be found elsewhere than in a stimulation of the rate of glutamine synthesis.

The addition of ammonium salts to the medium of photosynthesizing unicellular algae such as Chlorella pyrenoidosa causes shifts in metabolism such as decreased sugar formation and increased formation of certain amino acids and acids of the tricarboxylic acid (TCA) cycle (Hiller 1970, Kanazawa et al. 1970c, Kanazawa et al. 1972). In the dark, ammonia addition results in increased metabolism of sugar and increased flow of carbon into TCA acids and amino acids (Kanazawa et al. 1972), and increased carboxylation (Holm-Hansen et al. 1959).

The possible physiological role of ammonia in causing these shifts in metabolism in green algae is suggested by the fact that changes in the flow of carbon into biosynthetic end products during the cell cycle of synchronously-growing algae appear to be related to the changing ability of the cells to utilize nitrate over the cell cycle (Kanazawa et al. 1970a, Kanazawa et al. 1970b).

Similar profound effects of ammonia on the flow of carbon in the intermediary metabolism of photosynthetic cells from leaves of higher plants have been observed (Hammell et al. 1979, Lawyer et al. 1981, Pedersen et al. 1966, Platt et al. 1981, Woo and Canvin 1980). The physiological role of shifts in carbon metabolism in response to ammonia in higher plants may be related to the release of ammonia and its recycling during photorespiration (Keys et al. 1978, Lawyer et al. 1981). Nevertheless, the similarities between leaf cells and algae in the ammonia-induced shifts in carbon metabolism suggest that some mechanisms may be the same in both cases. The shifts in metabolism associated with ammonia appearance and use in the cytoplasm may be important in photosynthetic cells as a general regulatory mechanism. With poppy cells, all the effects were observed in the light (Paul et al. 1978), but stimulation of pyruvate kinase by ammonia was not observed in the dark

(Hammell et al. 1979). Since stimulation of PEP carboxylation was observed in the dark, different mechanisms may be responsible for the two effects.

The addition of ammonia to respiring Chlorella pyrenoidosa caused the steady-state level of ATP to drop about 20 % (Kanazawa et al. 1972). The change in ATP/ADP ratio or of energy charge indicated by this drop might be responsible for changes in the activities of one or more regulated enzymes. Plant pyruvate kinase (from seeds) has been reported to be regulated by energy charge (Duggleby and Dennis 1973). Stimulation of plant pyruvate kinase upon ammonia addition to green plant cells has been ascribed to decreased ATP levels (Givan 1973, Kanazawa et al. 1972, Paul et al. 1978). The lack of stimulation of pyruvate kinase in poppy leaf cells in the dark by ammonia might be related to the lack of increase in respiratory rate in that case (Hammell et al. 1979).

The stimulation of glutamine synthesis by ammonia would increase the use of ATP, and this might result in a decrease in ATP/ADP ratio and consequent regulatory effects (Bassham et al. 1981). A calculation of ATP utilization for this purpose in spinach cells treated with ammonia indicated that a significant change in ATP/ADP ratio should not result from this usage (Larsen et al. 1981). With the prokaryotic photosynthetic organism, Anabaena, ammonia stimulated dark CO₂ fixation (Ohmori et al. 1981). This stimulation could be abolished by addition of methionine sulfoximine, an inhibitor of glutamine synthesis. It was proposed that without inhibitor, ammonia-stimulated glutamine synthesis uses enough ATP to lower the energy charge, causing a stimulation of pyruvate kinase. Increased production of pyruvate could lead to an increased rate of formation of acetyl CoA. A higher level of acetyl CoA could activate PEPC, as that enzyme from some other types of cells is known to be activated by CoA (Ohmori et al. 1981). Blocking of glutamine synthesis with

methionine sulfoximine in higher plant cells leads rapidly to ammonia toxicity (Platt et al. 1981), however. Lack of enzyme activation under these circumstances could be due to excessive alkalinization.

Experiments comparing effects of ammonia with those induced by methylamine in light and dark in Chlorella pyrenoidosa were undertaken to examine the occurrence of regulatory effects in the absence of stimulated glutamine synthesis, but without toxicity due to ammonia accumulation. In addition, the transient changes in labelling rates of various amino acids and carboxylic acids upon addition of ammonia or methylamine have been more carefully examined for clues which might indicate the sequence of events involved in the activations of PK and PEPC. The results indicate that greatly accelerated glutamine synthesis is not required for the principal regulatory effects to be seen. The transient changes in labelling rates lend support to the proposal that the primary mechanism is via an increase in pH leading to PEPC activation. The stimulation in PK and the increased rate of triose phosphate oxidation remain to be explained. They might be triggered in some way by the large changes in metabolite concentrations resulting from PEPC activation, or by lowered ATP/ADP ratios caused by ammonia effects other than increased glutamine synthesis.

MATERIALS AND METHODS

Chlorella pyrenoidosa was cultured aseptically at 25 C in a 1 l vertical culture tube (Bassham and Calvin 1957) with an internal cooling well and surrounded by a bank of eight 20 watt fluorescent lights giving an average light intensity incident to the culture tube of $200 \text{ uE m}^{-2} \text{ sec}^{-1}$. Cell density was kept constant at 0.5 % (packed cell volume/total suspension volume) by adding culture medium (Kanazawa et al. 1970) automatically.

A 200 ml portion of the cell suspension was removed from the culture tube, and the cells were separated from the medium by low speed centrifugation for three min. They were then washed by resuspension in 50 ml 10 mM KH_2PO_4 buffer, centrifuged, and resuspended to 1 % (v/v) in the same buffer. An aliquot sample was analyzed for chlorophyll content (Arnon 1949).

For each experiment, 0.95 ml of this 1 % algal suspension was placed in a 10 ml round bottom flask fitted with a serum stopper. The flask was mounted in one of the holders on the rotary shaker-water bath illuminated from below ($650 \text{ uE m}^{-2} \text{ s}^{-1}$) (Jensen et al. 1966). The water bath temperature was controlled at 22 C. Then 0.05 ml of 0.133 M NaHCO_3 solution labelled with ^{14}C (14.74 uCi/umole) was injected through the serum stopper into the cell suspension, giving a final concentration of 6.65 mM NaHCO_3 .

For the "light" experiments, the time indicated in the RESULTS began with the bicarbonate addition. Samples (50 ul) were removed at the times indicated by withdrawal with a syringe needle inserted through the serum cap. Samples were added to 0.400 ml methanol in stoppered vials for killing. After several samples had been removed, 40 ul of either NH_4Cl or $\text{CH}_3\text{NH}_3\text{Cl}$ solution (final concentration, 1.0 mM) was added to a flask. Further samples were removed from these flasks and from the control flasks as indicated. For "dark" experiments, the light period (photosynthesis) was for 15 min, then the light was turned off (time 0) and samples were subsequently taken at the times indicated and killed as just described.

From each sample in methanol, a 20 ul aliquot portion was removed, acidified, dried, and the acid-stable radioactivity determined to give total ^{14}C fixation rates. A 200 ul aliquot portion was removed and spotted on paper for analysis by two dimensional paper chromatography (Pedersen et al. 1966). After separation, areas of paper containing radioactive compounds were cut

out, cut up and placed in scintillation vials, and the ^{14}C contents were determined by scintillation counting (Larsen et al. 1981). The amount of ^{14}C labelling of each compound was calculated and expressed as ug-atoms ^{14}C per mg of chlorophyll. The uC of ^{14}C found in each compound per mg chlorophyll in the sample from which it was derived divided by the specific radioactivity of the bicarbonate used (14.74 uC/umole) gives ug-atoms ^{14}C per mg chl.

RESULTS

The purpose of the present study was principally to compare effects of methylamine addition with effects of ammonia addition. The data from the experiment with ammonia addition in the light (not shown in every case) were in all cases comparable in quality to the data shown. Both the qualitative and quantitative changes in labelling upon ammonia addition were generally similar for the light and dark experiments.

Glutamine. Ammonia addition caused a large increase in the labelling of glutamine in the light (data not shown) and in the dark (Fig 1), as observed in past studies (Kanazawa et al. 1970c, Kanazawa et al. 1972). With methylamine, in the light or the dark, glutamine labelling is only slightly accelerated. Thus, the changes in labelling rate seen with ammonia addition are not reproduced by the addition of methylamine. Perhaps equally significant is the fact that methylamine does not inhibit the synthesis of glutamine.

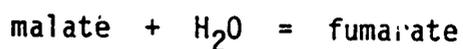
Glutamate. The effects on glutamate labelling of adding ammonia and of adding methylamine are similar in the light and in the dark (Figs 2,3). The eventual labelling of glutamate seems to be somewhat higher with methylamine than with ammonia. In the light, there is a delayed stimulation of glutamate synthesis in both cases. The level of labelled glutamate before additions is higher in the dark than in the light, and rises to much higher levels in the

dark (compared to the dark control) following the addition of either ammonia or of methylamine.

Citrate. The labelling of citrate rises very rapidly in the dark, beginning with the first and second point after the additions of ammonia or methylamine. (Fig 4). The post-addition rise in citrate labelling is about twice as much with methylamine as with ammonia.

In the light, the labelling rises rapidly after about one minute, following the addition of methylamine or of ammonia. The increase with methylamine is greater than with ammonia (Fig 5). The immediate labelling increase following additions, followed by a pause before further increase, though based on a limited number of data points, may be significant, as it occurs with both ammonia and methylamine (Fig. 5a).

Malate, Fumarate. Addition of ammonia or of methylamine, in light or dark, causes immediate and large increases in the labelling of malate and fumarate (Figs 6-9). The labelling of malate is in all cases about four times that of fumarate, so that when the curves for malate and for fumarate ($\times 4$) are compared, they are nearly superimposable. When the labelling is increasing rapidly, the fumarate labelling curve lags slightly behind the malate curve, as expected if fumarate is formed from malate. If the malate and fumarate pools were in true equilibrium at pH 7 and 298 C at a ratio malate/fumarate = 4, the the calculated free energy change ΔG for the reaction



would be +0.82 Kcal. The actual ΔG is reported to be +0.88 Kcal (Krebs 1953). This data suggests that the labelled, actively turning-over pools (which quite

likely are smaller than the total pools) of malate and fumarate are close to being in equilibrium with each other.

In the light, addition of methylamine causes labelling of both malate and fumarate to rise about twice as fast and as far as does the addition of ammonia. In the dark, the increase in labelling of these acids with methylamine is over three times as much as with ammonia addition.

Aspartate. In the light, aspartate label rises rapidly after addition of ammonia and much less rapidly after addition of methylamine (Fig 10). In the dark, the rise in aspartate labelling following ammonia addition is about the same as following addition of methylamine (Fig 11).

Alanine. Addition of ammonia, in light or dark, causes a rapid rise in the labelling of alanine, although the effect is more immediate in the dark and starts from a higher control level. With methylamine, the initial rises are similar to those with ammonia, but the ultimate levels reached are considerably lower (Figs 12,13).

Phosphoenolpyruvate. With added ammonia, in light or in dark, there is a rapid and substantial drop in level of labelled PEP. With added methylamine, there also is some drop in PEP level, but it is smaller (Figs 14,15).

Other Compounds. The labelling of a number of other compounds before and after additions of ammonia and of methylamine were examined. These included sugar diphosphates, hexose monophosphates, sucrose, uridine diphosphoglucose, phosphoglycerate, serine and glycolate. These data for ammonia added in light and dark generally agreed with the results seen on our previous studies. Differences between the consequences of addition of methylamine and of ammonia seemed to be generally trivial, except perhaps that in the light,

methylamine addition caused a smaller decrease in sucrose labelling (Fig 16) and a larger decrease in sugar diphosphate labelling than ammonia addition (data not shown).

An examination of changes in levels of amino acids 8 min after the additions, and comparison with the controls in each experiment (Table 1) shows that the increase in labelling of glutamate was greater with methylamine than with ammonia addition, in the light experiments. Labelling of aspartate, alanine, and serine increased less with methylamine. This pattern of relative changes in amino acid labelling is helpful in interpreting the probable relative effects of additions of ammonia and of methylamine on glutamate pool sizes or activity of transaminases as discussed later.

DISCUSSION

Glutamine Synthesis. With the addition of the ammonia analog, methylamine, most of the rapid changes in rates of flow of carbon from sugar phosphates into C_3 , C_4 , and C_5 acids and amino acids seen with ammonia are reproduced. The exception is that there is no large stimulation in glutamine synthesis. It is thus concluded that greatly accelerated glutamine synthesis is not required to trigger the acceleration of reactions mediated by PEPC and PK. It may also be inferred that oxidation of triose phosphates to PEP is accelerated upon methylamine addition, and that a large increase in glutamine synthesis is not required for this major shift in the metabolic fate of sugar phosphates upon addition of ammonia (or methylamine).

It is noteworthy that with the addition of methylamine, glutamine labelling in the light is not inhibited but in fact is slightly accelerated. We interpret this slight increase in labelling rate to an increased specific labelling of glutamate due to the increased rate of flow of ^{14}C into

acids of the TCA cycle as a consequence of PK and PEPC stimulation. The slight increase in rate of glutamine labelling suggests that the normal reaction (with ammonia) is not inhibited by the presence of methylamine.

Depending on the detailed mechanism of the glutamine synthetase reaction including any capacity of the enzyme to interact with methylamine in place of ammonia, it might be that methylamine could participate in a futile reaction leading to the hydrolysis of ATP without any net change in carbon compounds. The postulated phosphoryl glutamate intermediate which reacts with ammonia to give glutamine conceivably might be hydrolyzed in the presence of methylamine rather than forming N-methyl glutamine (which we don't find). This reaction pathway would result in the net hydrolysis of ATP. The lack of any inhibition of glutamine labelling in the presence of methylamine makes such an interaction appear unlikely, however.

Phosphoenolpyruvate Carboxylation. The stimulation of the labelling of the tricarboxylic acid cycle compounds citrate, malate, and fumarate is as large or larger with methylamine addition as with ammonia addition. In the present study, we have examined closely the early responses to the additions. The immediate increases seen in labelling of malate and of fumarate with light and dark and with both ammonia and methylamine strongly indicate a rather direct effect, rather than one mediated by a primary change in concentration of some other metabolite.

The similar labelling curves for malate and fumarate, but with a slight lag in the fumarate curve during periods of most rapid labelling indicate that the direction of labelling is from malate to fumarate (reversed direction of the TCA cycle) rather than from fumarate to malate. Clearly the acceleration of PEP carboxylation is too great for the resulting oxaloacetate to be used entirely for condensation with acetyl CoA to give citrate.

In the light, addition of either ammonia or of methylamine causes a sustained rapid increase in labelling of citrate after about 1 min (by the fourth data point following addition). The contrast with malate and fumarate labelling suggests that, while PEP carboxylation is immediately stimulated, citrate labelling (in the light) cannot increase continuously until the PK reaction has been stimulated, and carbon has begun to flow at an increased rate through the pools of pyruvate and acetyl CoA. In fact, the first data point after additions shows an increase in citrate label in both cases in the light, followed by a pause (see Fig 5a). As the rate of PEP carboxylation increases immediately following additions of ammonia or of methylamine, the resulting oxaloacetate may react with all the available acetyl CoA. In the dark, the rise in citrate is immediate following the additions, suggesting that there is a larger acetyl CoA pool already. This might be expected under conditions of respiratory metabolism.

While malate, fumarate and citrate increased more with methylamine addition than with ammonia, the increase in labelling of aspartate in the light was less. In the dark the increases, after a delay, were about the same. The rate of aspartate labelling depends on oxaloacetate acid pool size and specific activity, glutamate pool size, and glutamate-oxaloacetate aminotransferase activity. None of these were directly measured.

The higher eventual labelling of glutamate in the light with methylamine as compared with ammonia may mean that the glutamate pool became larger with methylamine, due perhaps to inhibition of glutamate aminotransferases. This possibility seems to be supported by the fact that in the light, the increases in labelling of aspartate, serine, and alanine seen with methylamine addition (compared with the corresponding control) were much less than with ammonia (Table 1).

Conversion of phosphoenolpyruvate to pyruvate. PEP labelling is

affected positively by increase in the rate of conversion of triose phosphates to phosphoglycerate and negatively by increased PEP carboxylation and by the conversion to pyruvate. As already discussed, the evidence indicates that PEP carboxylation is, if anything, more stimulated with methylamine than with ammonia. The fact that PEP labelling declines much less with methylamine, and then is restored, suggests that either triose phosphate oxidation is more stimulated (with methylamine), pyruvate kinase is less stimulated, or both. The rapid increase in alanine labelling with methylamine addition, in light or dark, clearly indicates that the conversion of PEP to pyruvate has increased. That the eventual increase is much less (in both light and dark) may indicate a smaller stimulation of pyruvate kinase with methylamine addition (compared with ammonia) or it might be due to some methylamine inhibition of aminotransferase, as discussed above. In any event, it is clear that PEP to pyruvate conversion rate was increased in the presence of methylamine.

Conversion of triose phosphates to phosphoenolpyruvate. Evidence already presented indicates that the addition of methylamine resulted in a large stimulation in PEP carboxylation and in an increased rate of conversion of PEP to pyruvate. Nevertheless, the level of labelled PEP declined less after methylamine addition than after ammonia addition, both in light and in dark. The PEP pool size (which is approximately indicated by its label in these studies, since it is directly derived from photosynthate) is small and ^{14}C must pass rapidly through it. It may be inferred, therefore, that the rate of formation of PEP from sugar phosphates is greatly increased upon addition of methylamine, perhaps more so than on the addition of ammonia. This increased flow of carbon from sugar phosphates into PEP seems to have been less at the expense of sucrose formation with methylamine addition than when ammonia was

added (Fig 16). The alternate source of photosynthate carbon would be starch in the chloroplasts.

Possible regulatory mechanisms. Accelerated glutamine synthesis per se is apparently not required for the manifestation of the other regulatory effects previously seen with ammonia addition. Stimulation of PEP carboxylation by an increase in cytoplasmic pH with increased ammonia in the cytoplasm seems plausible (Hammell et al. 1979), considering that a similar or even greater apparent stimulation of PEP carbpxylation is seen when the ammonia analog methylamine is added in place of ammonia.

If the primary change is activation of PEP carboxylation via a pH change, the resulting changes in concentrations of other metabolites such as the carboxylic acids and certain amino acids might be able to activate other enzymes. Changes in levels of ATP and ADP may be occurring also, as found previously with ammonia addition (Kanazawa et al. 1970c, Kanazawa et al. 1972). If so, they are not due to greatly accelerated glutamine synthesis. There may be some kind of uncoupling of oxidative phosphorylation from electron transport in algae, despite the finding that higher plant mitochondria do not exhibit such uncoupling in response to ammonia (Hammell et al. 1979). Detailed studies of the kinetic and regulatory properties of the isolated and purified PK and other enzymes from Chlorella will be required before the regulatory mechanisms can be fully identified.

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Table 1

AMINO ACID LABELLING AS AFFECTED BY AMMONIA AND METHYLAMINE

ug-atoms ^{14}C per mg chl at 8 min after additions

		NH_3			CH_3NH_2		
		+	-	diff.	+	-	diff.
Light	Glu	0.89	0.21	0.68	1.26	0.40	0.86
	Asp	0.89	0.28	0.61	0.64	0.49	0.15
	Ala	1.55	0.18	1.37	0.66	0.18	0.43
	Ser	0.47	0.22	0.25	0.28	0.23	0.05
Dark	Glu	1.38	0.82	0.56	2.02	1.38	0.64
	Asp	0.94	0.44	0.50	1.10	0.63	0.47
	Ala	1.56	0.55	1.01	0.93	0.50	0.43
	Ser	0.37	0.28	0.09	0.38	0.29	0.09

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FIGURES

FIGURE 1 Effects of ammonia and of methylamine on the labelling of glutamine.

In Figures 1-5a and 10-16, triangles represent the flask in which ammonia was added at the time indicated, circles represent the flask to which methylamine was added, and crosses represent the control flasks; open symbols are for light experiments and closed symbols are for dark experiments. The control in Figure 1 was for a darkened flask.

FIGURE 2. Effects of ammonia and of methylamine on labelling of glutamate (light).

FIGURE 3. Effects of ammonia and of methylamine on labelling of glutamate (dark).

FIGURE 4. Effects of ammonia and of methylamine on labelling of citrate (dark).

FIGURE 5. Effects of ammonia and of methylamine on labelling of glutamate (light)

FIGURE 5a. Effects of ammonia and of methylamine on labelling of citrate (light, expanded near time of additions).

FIGURE 6. Effects of ammonia on labelling of malate and fumarate (light).

In Figures 6-9, circles represent malate labelling, triangles represent fumarate labelling.

FIGURE 7. Effects of methylamine on labelling of malate and fumarate (light).

FIGURE 8. Effects of ammonia on labelling of malate and fumarate (dark).

FIGURE 9. Effects of methylamine on labelling of malate and fumarate (dark).

FIGURE 10. Effects of ammonia and methylamine on labelling of aspartate (light).

- FIGURE 11. Effects of ammonia and methylamine on labelling of aspartate (dark).
- FIGURE 12. Effects of ammonia and methylamine on labelling of alanine (light).
- FIGURE 13. Effects of ammonia and methylamine on labelling of alanine (dark).
- FIGURE 14. Effects of ammonia and methylamine on labelling of PEP (light).
- FIGURE 15. Effects of ammonia and methylamine on labelling of PEP (dark).
- FIGURE 16. Effects of ammonia and methylamine on labelling of sucrose (light).

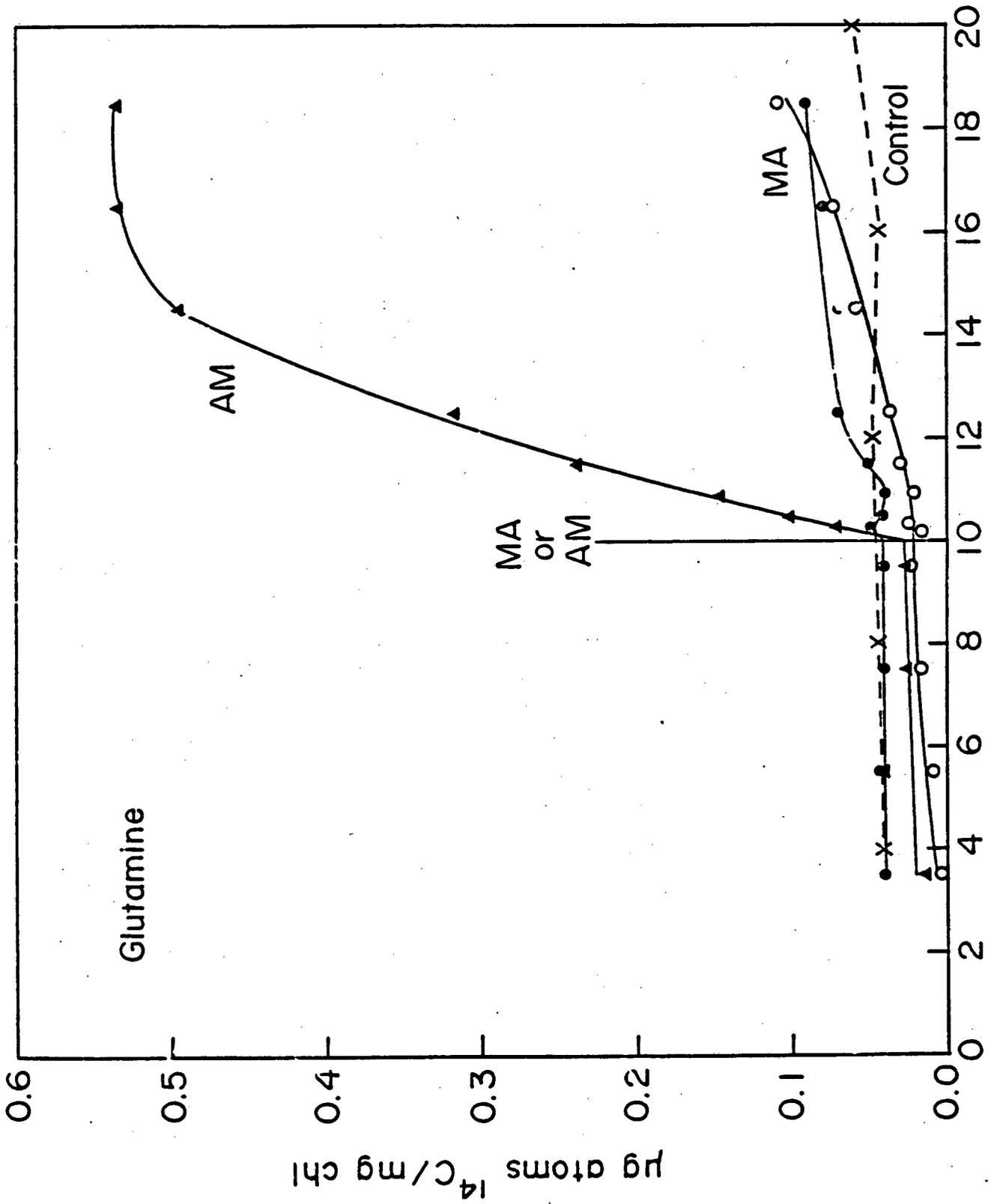


Figure 1

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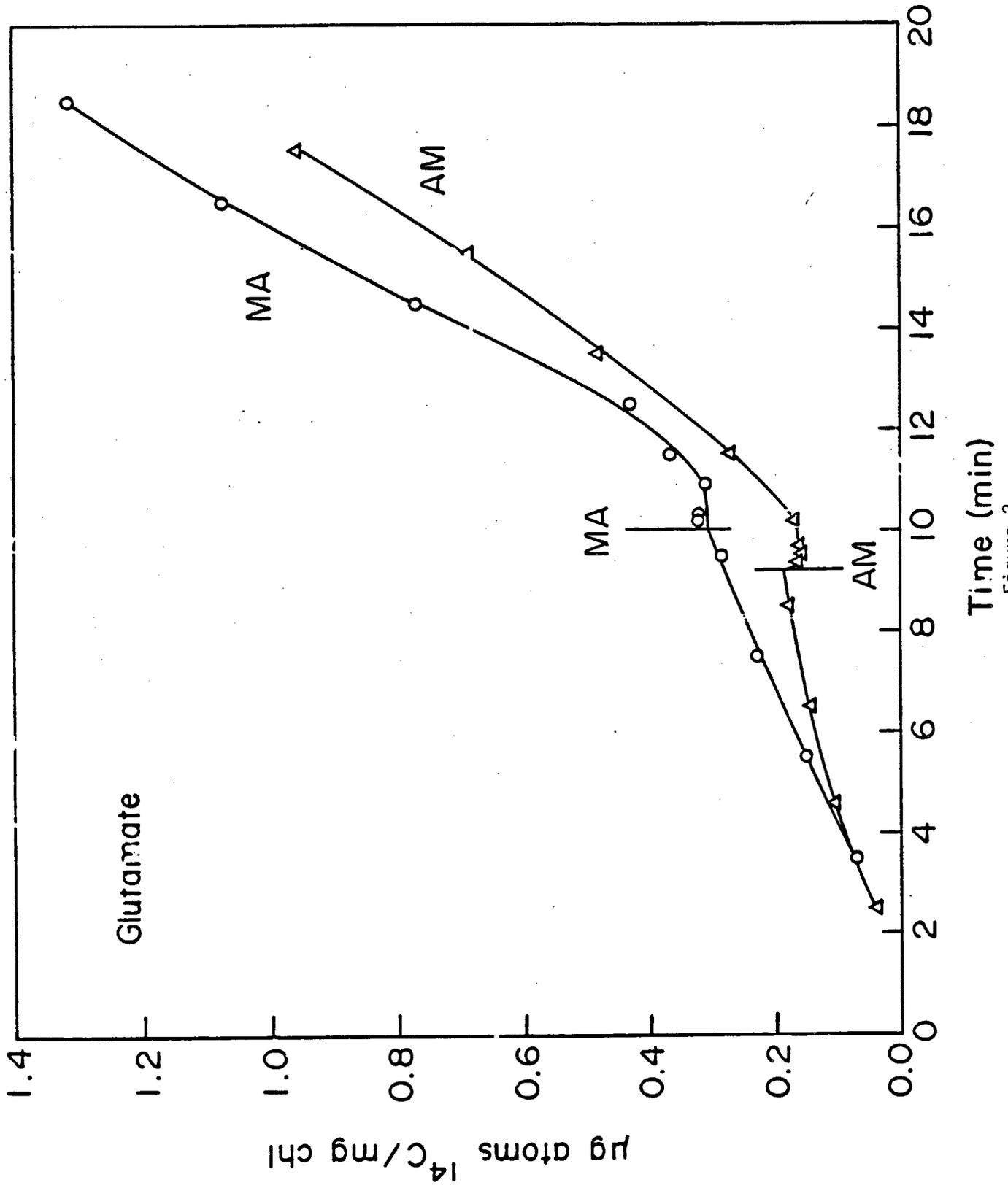
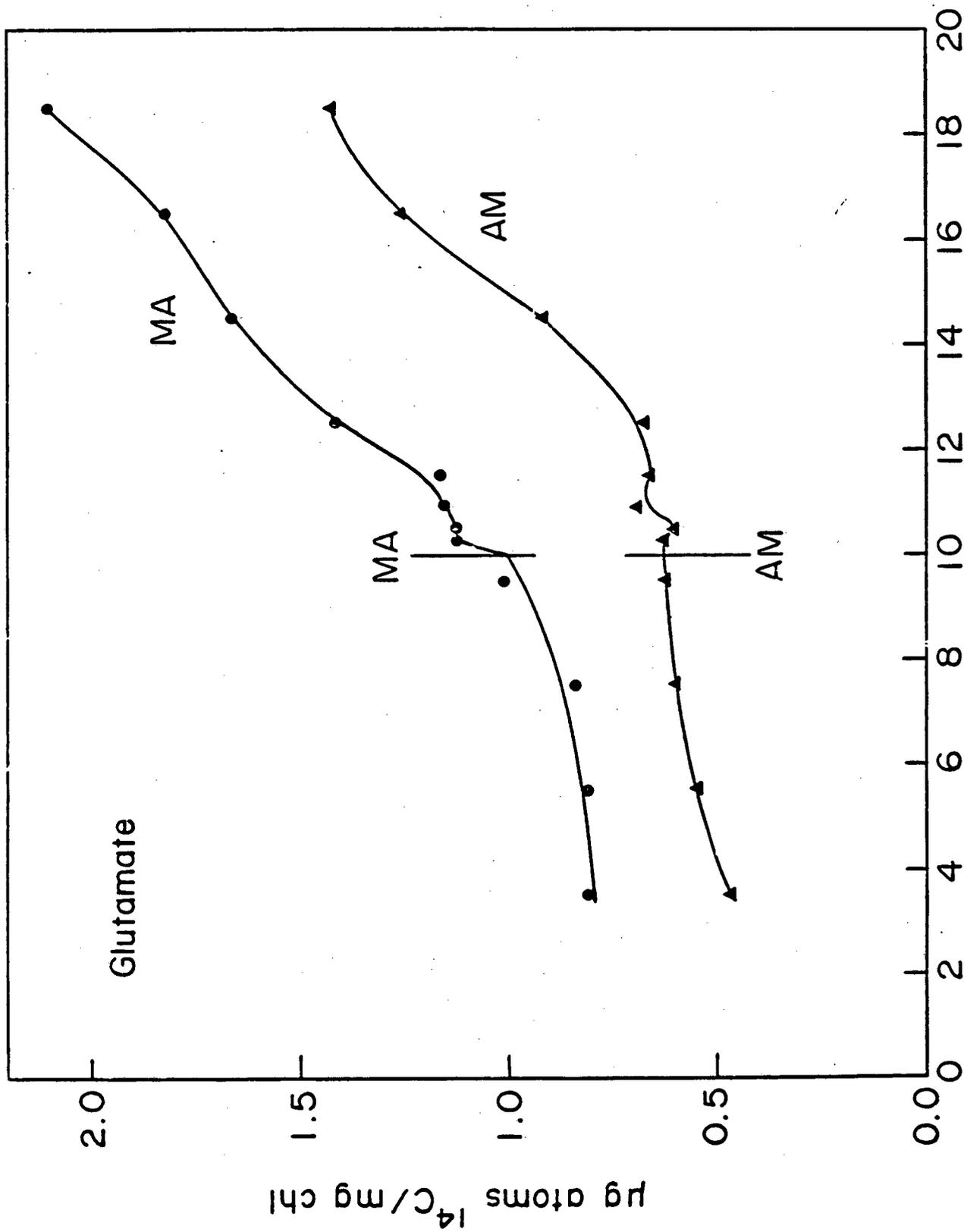


Figure 2



Time (min)
Figure 3

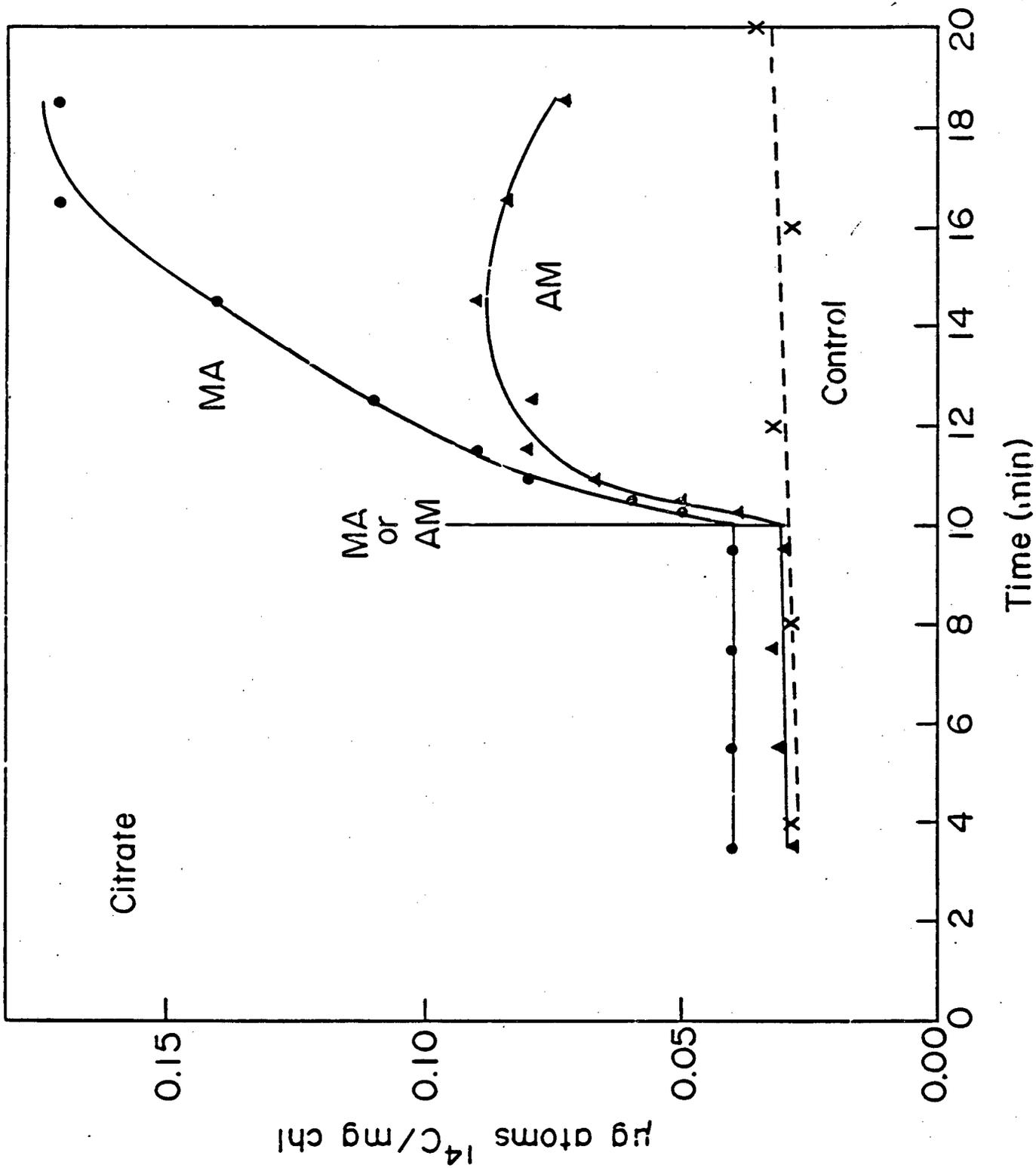


Figure 4

Fig. 4

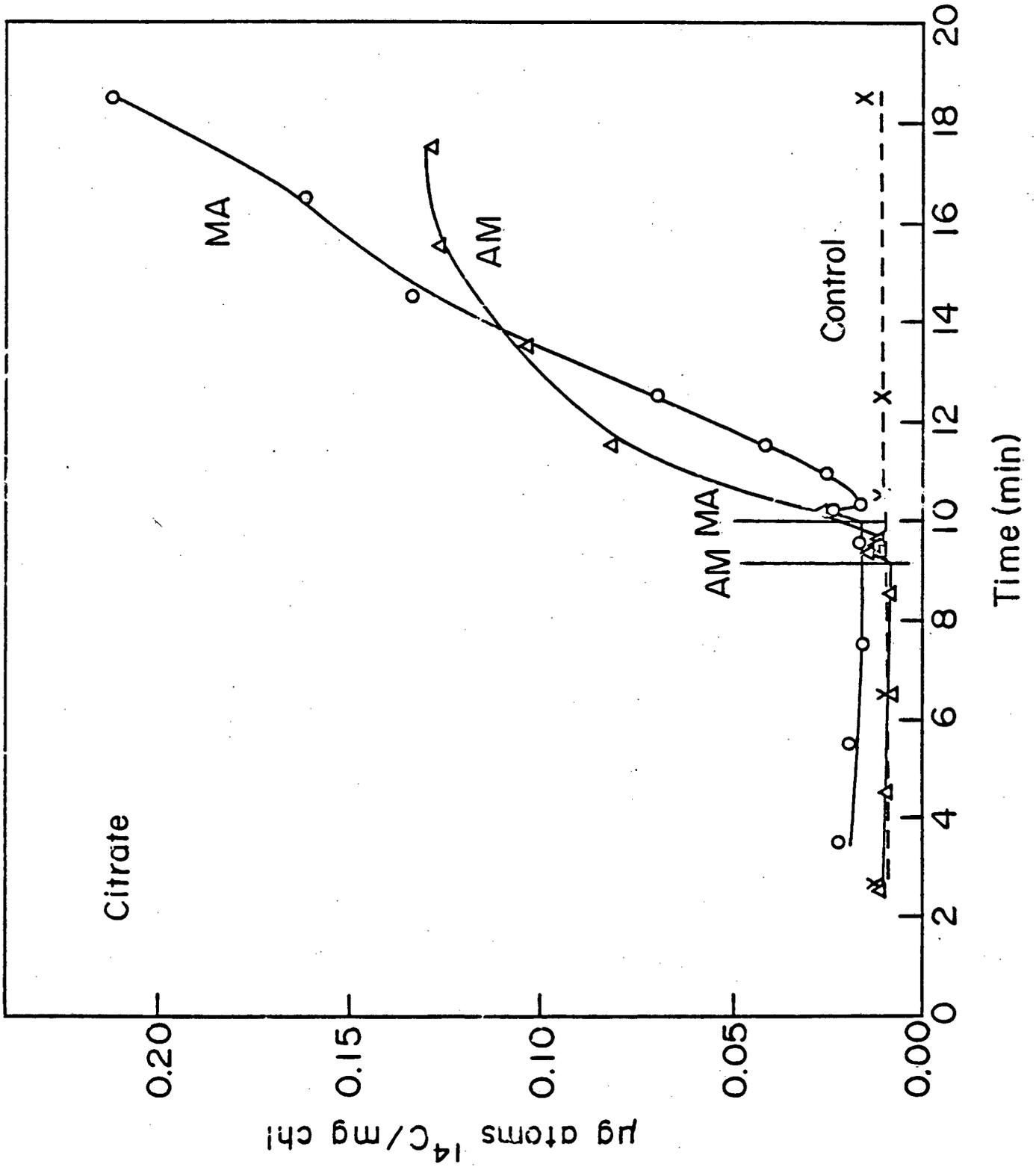


Figure 5

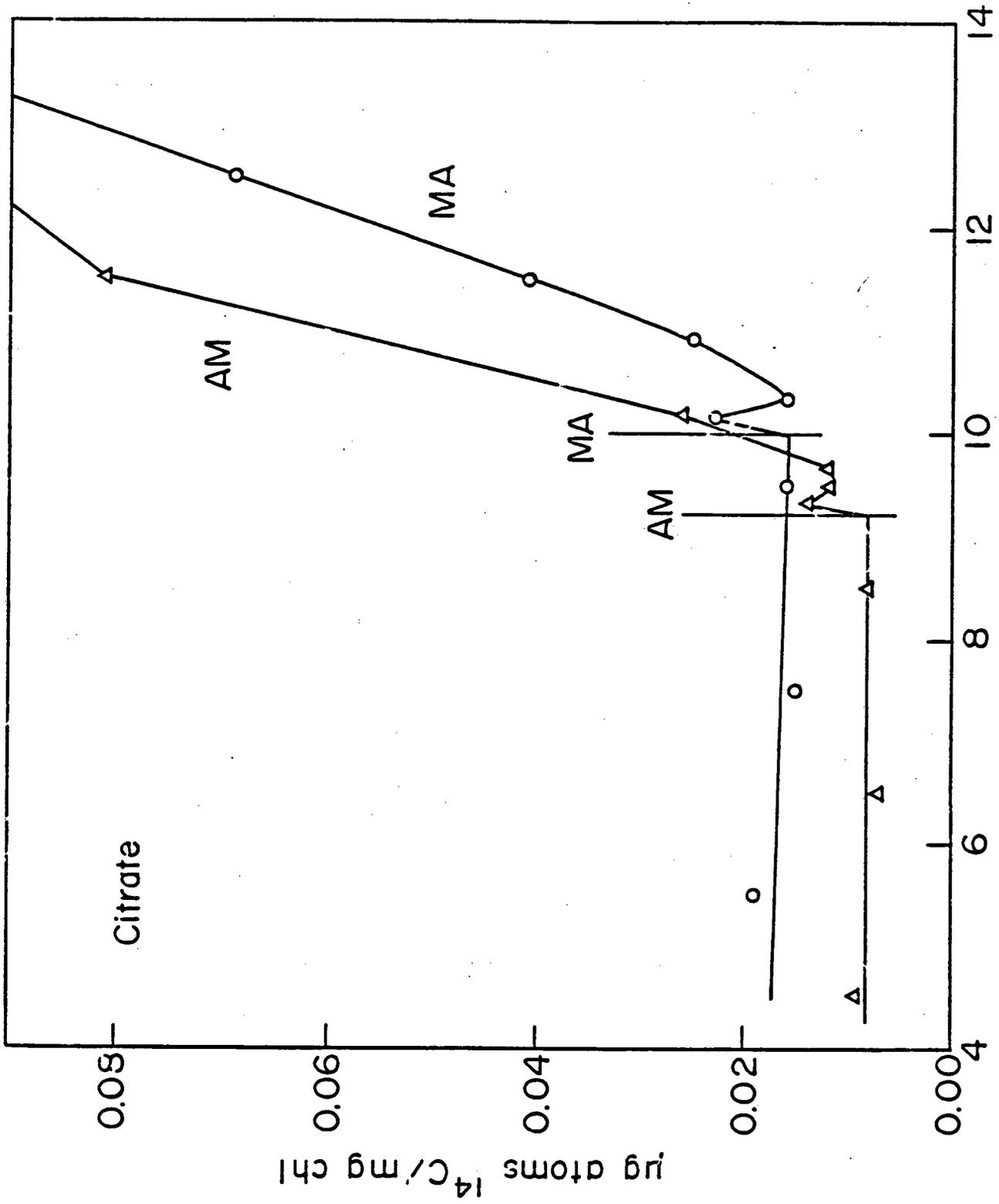
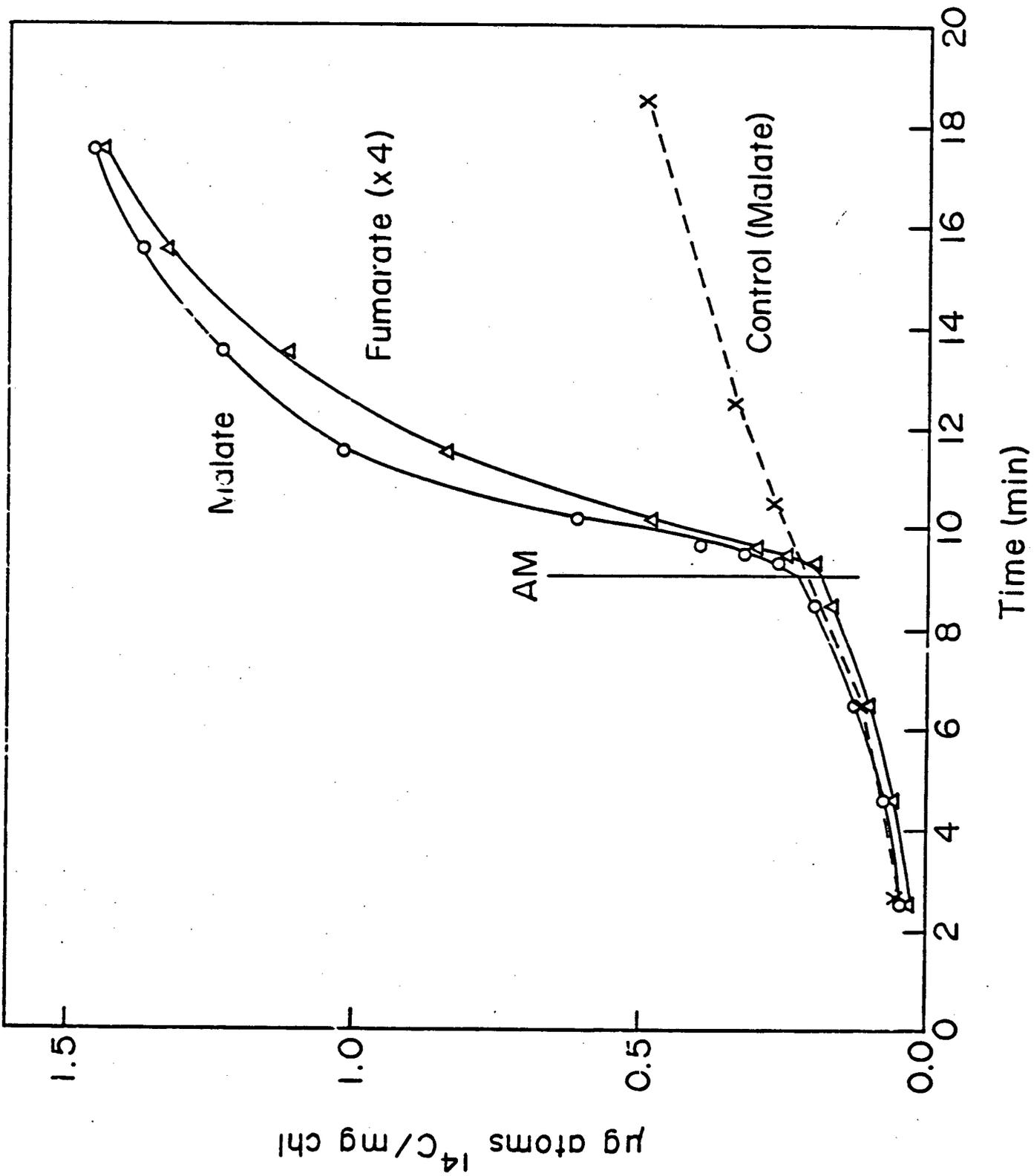


Figure 5 a

Fig. 5a



Time (min)
Figure 6

Fig. 6

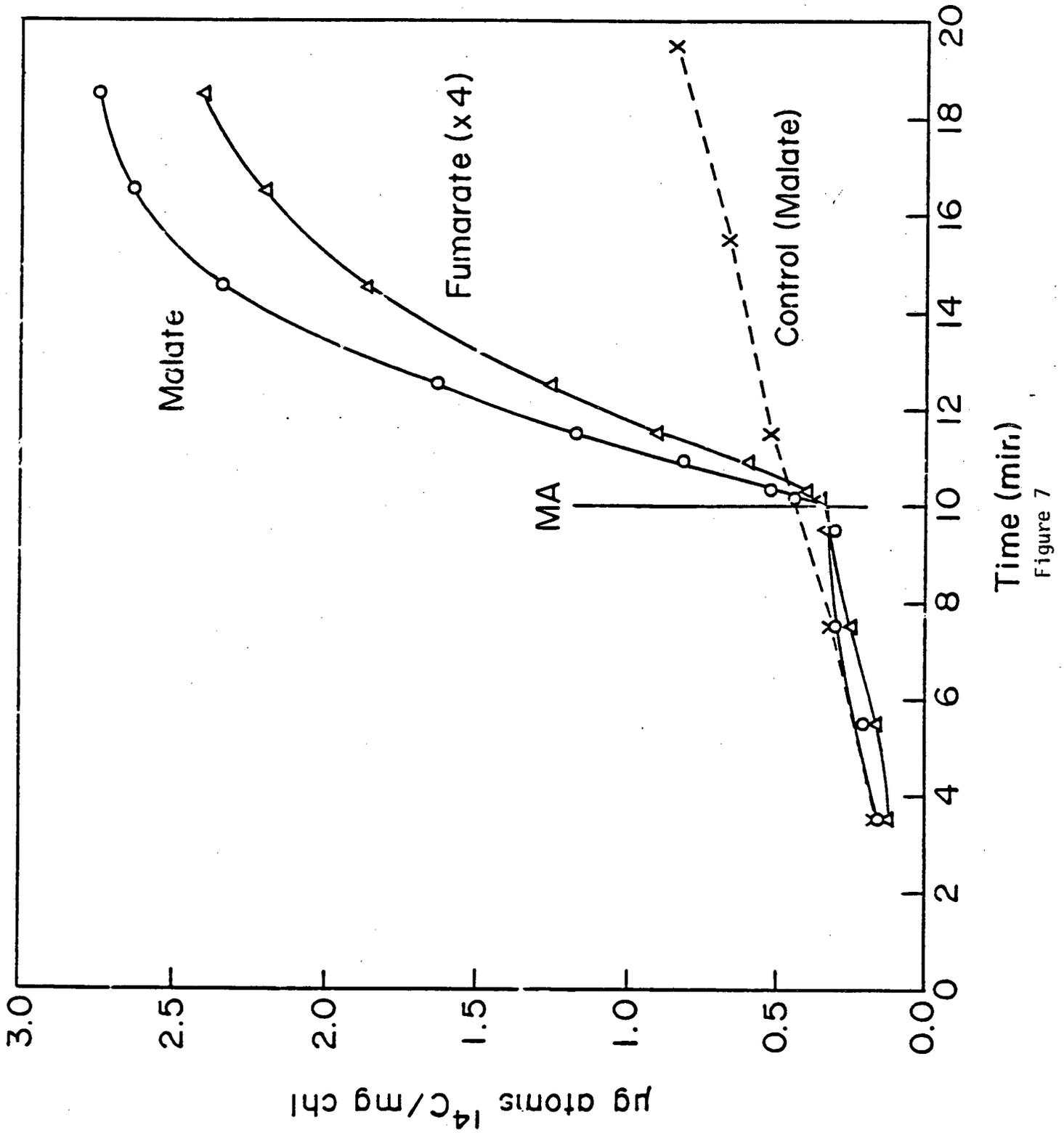


Figure 7

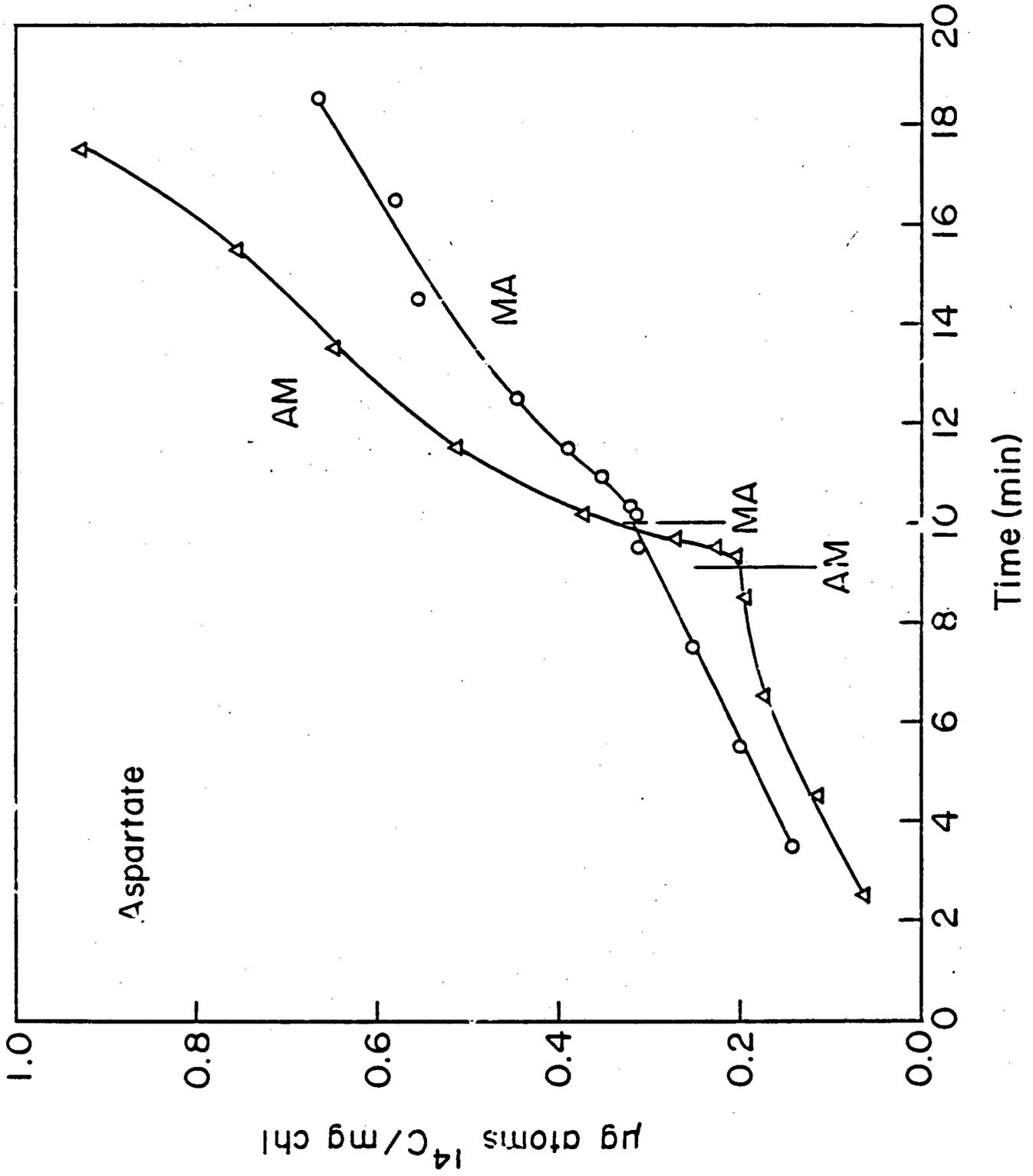


Figure 10

Fig. 10

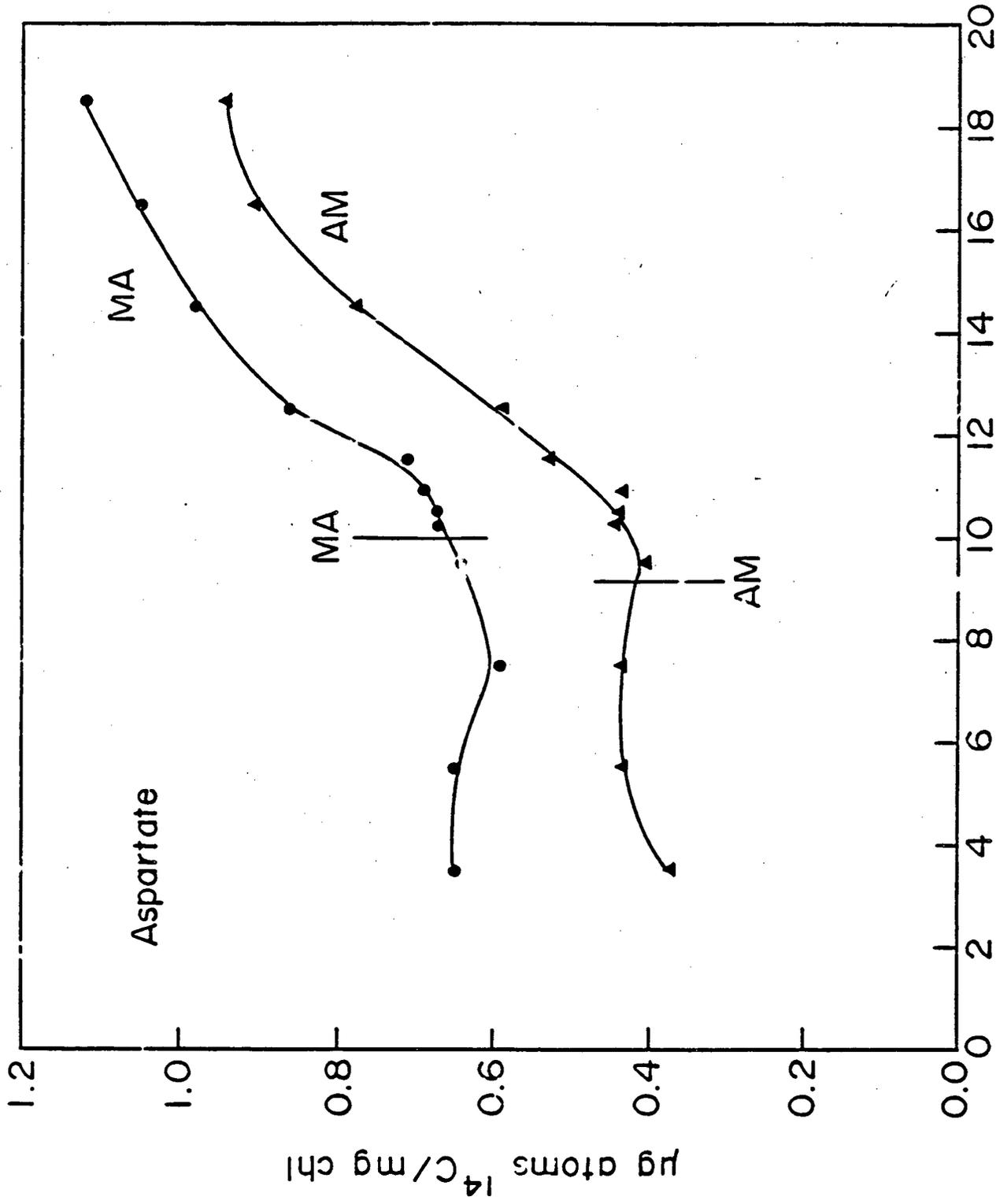


Figure 11

Fig. 11

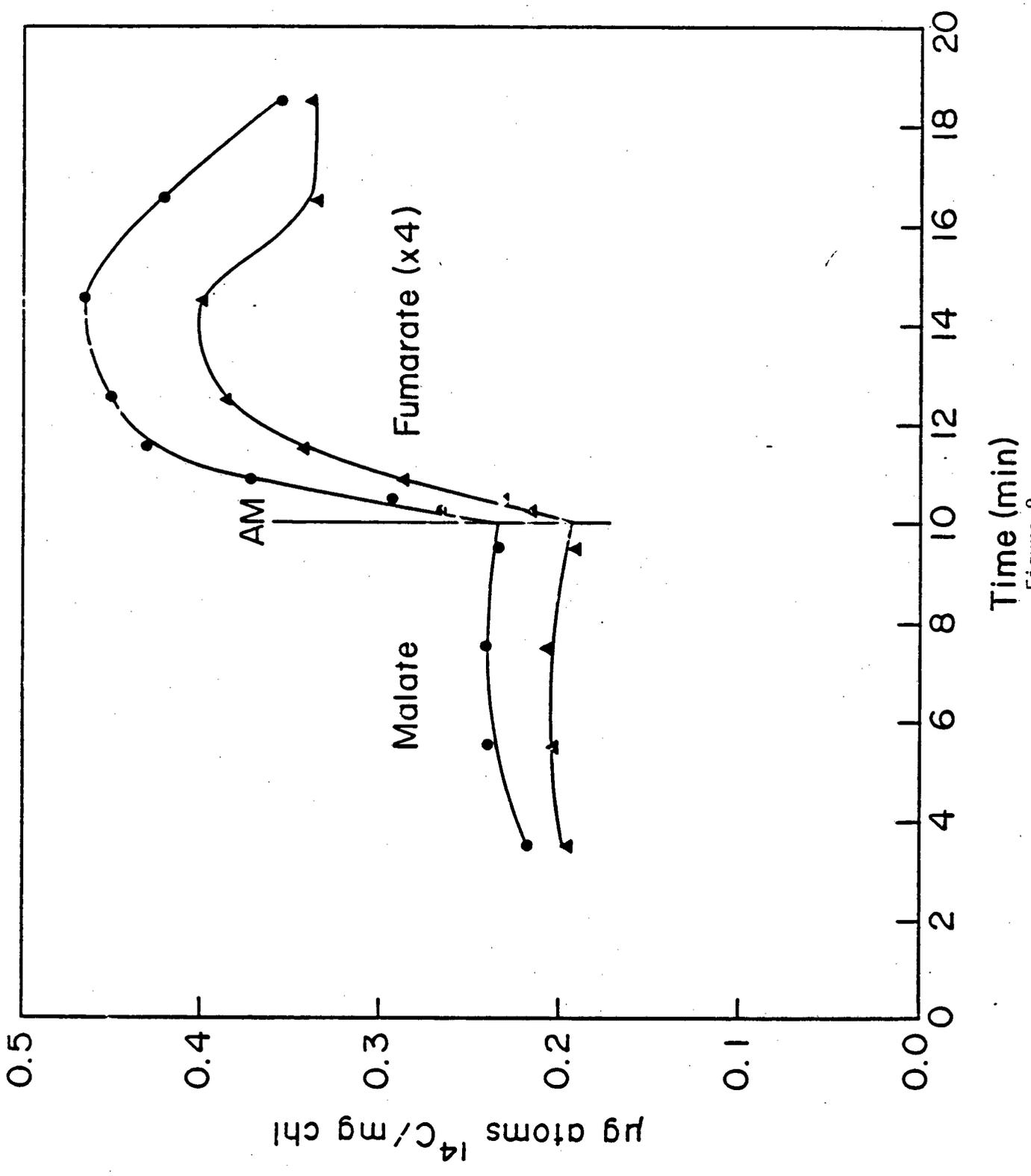
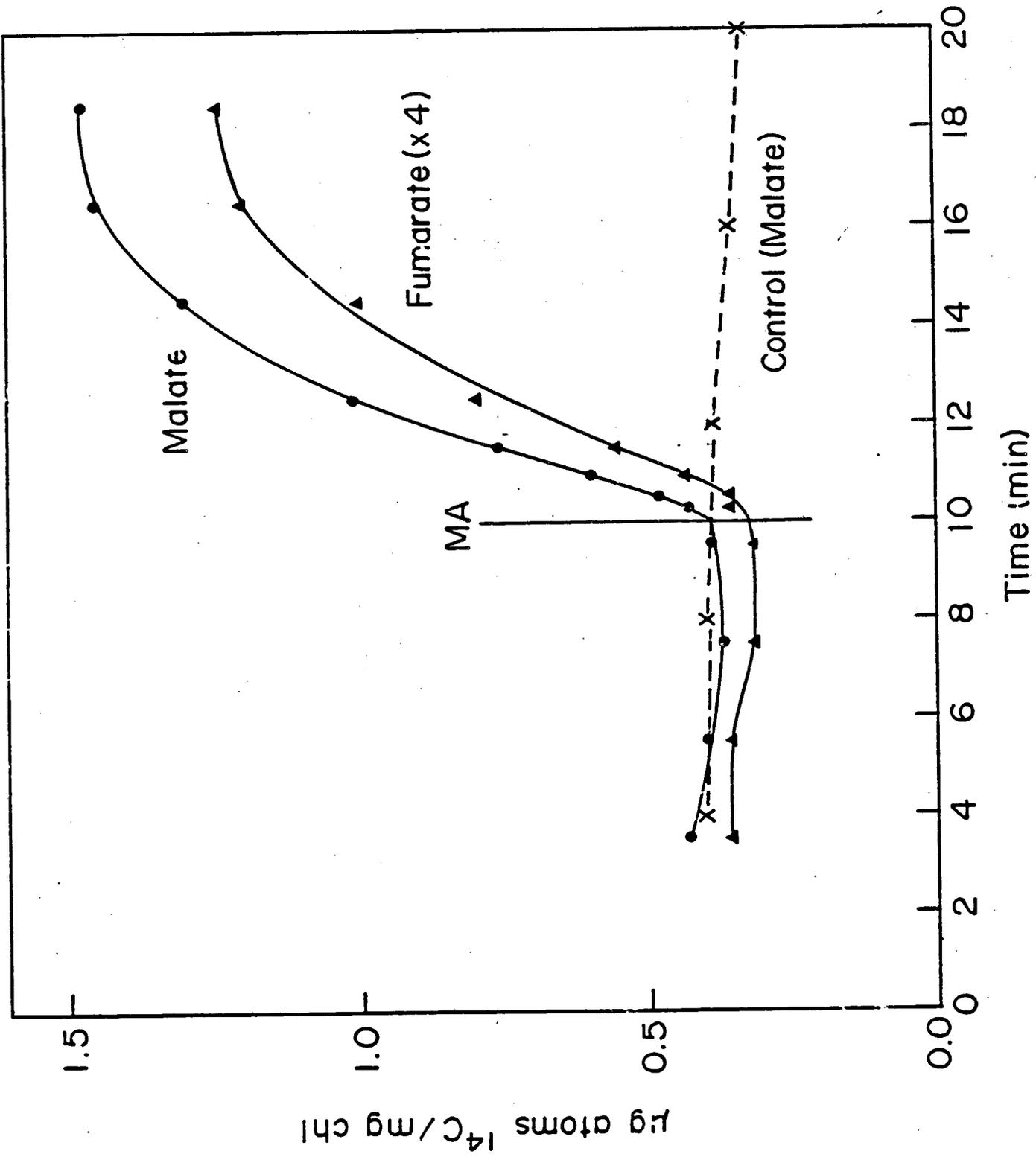


Fig. 8



Time (min)
Figure 9

Fig. 9

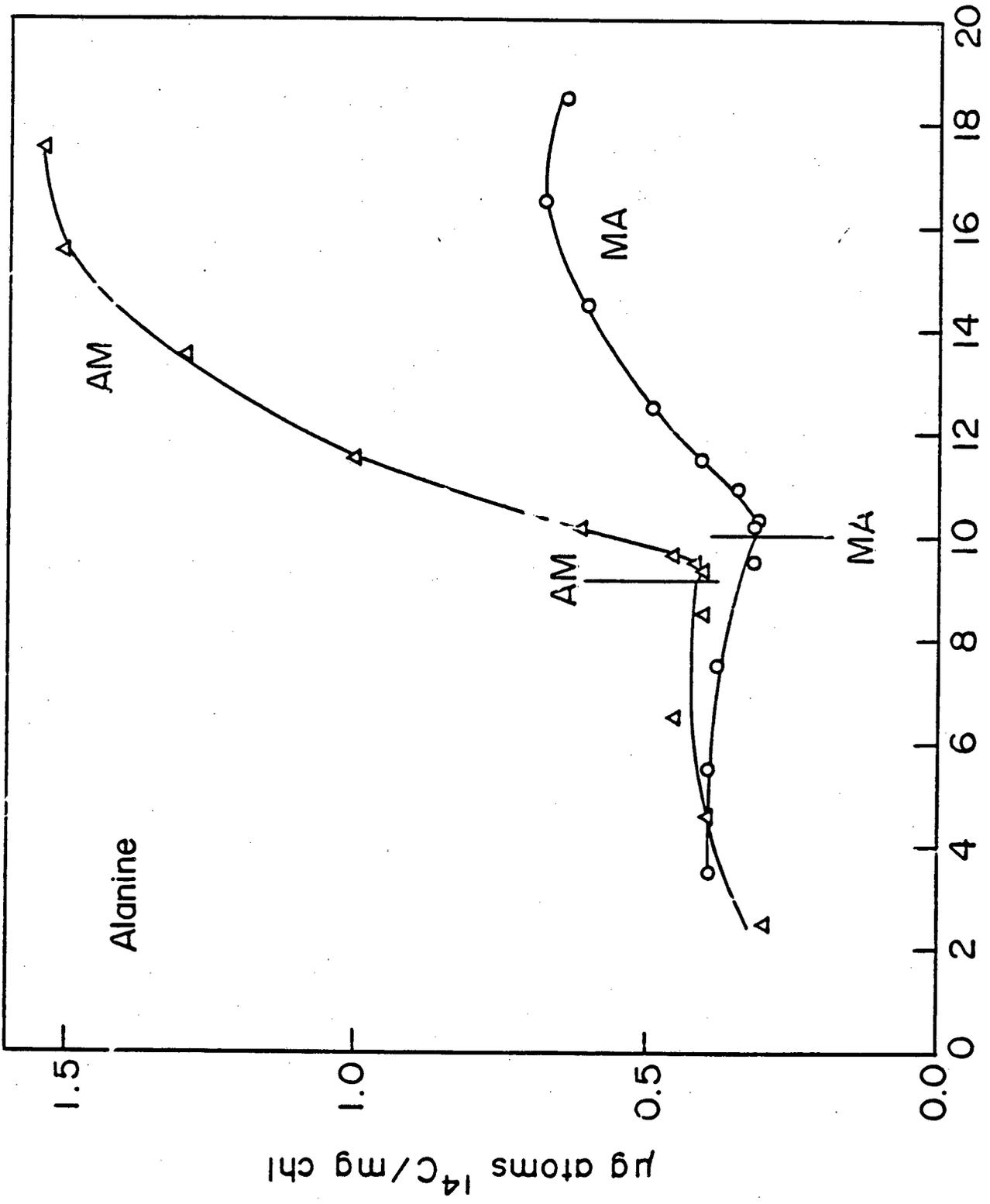


Figure 12

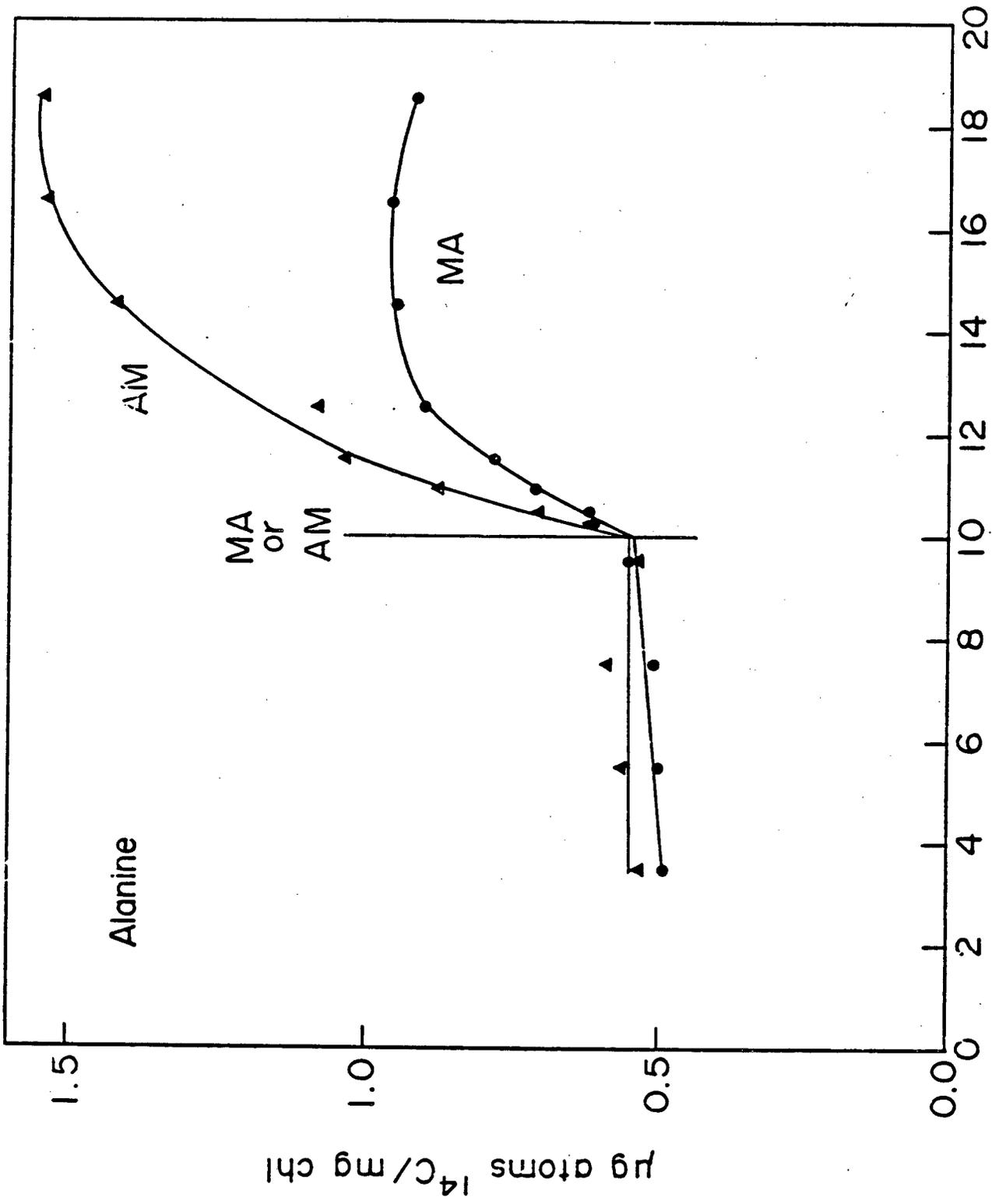
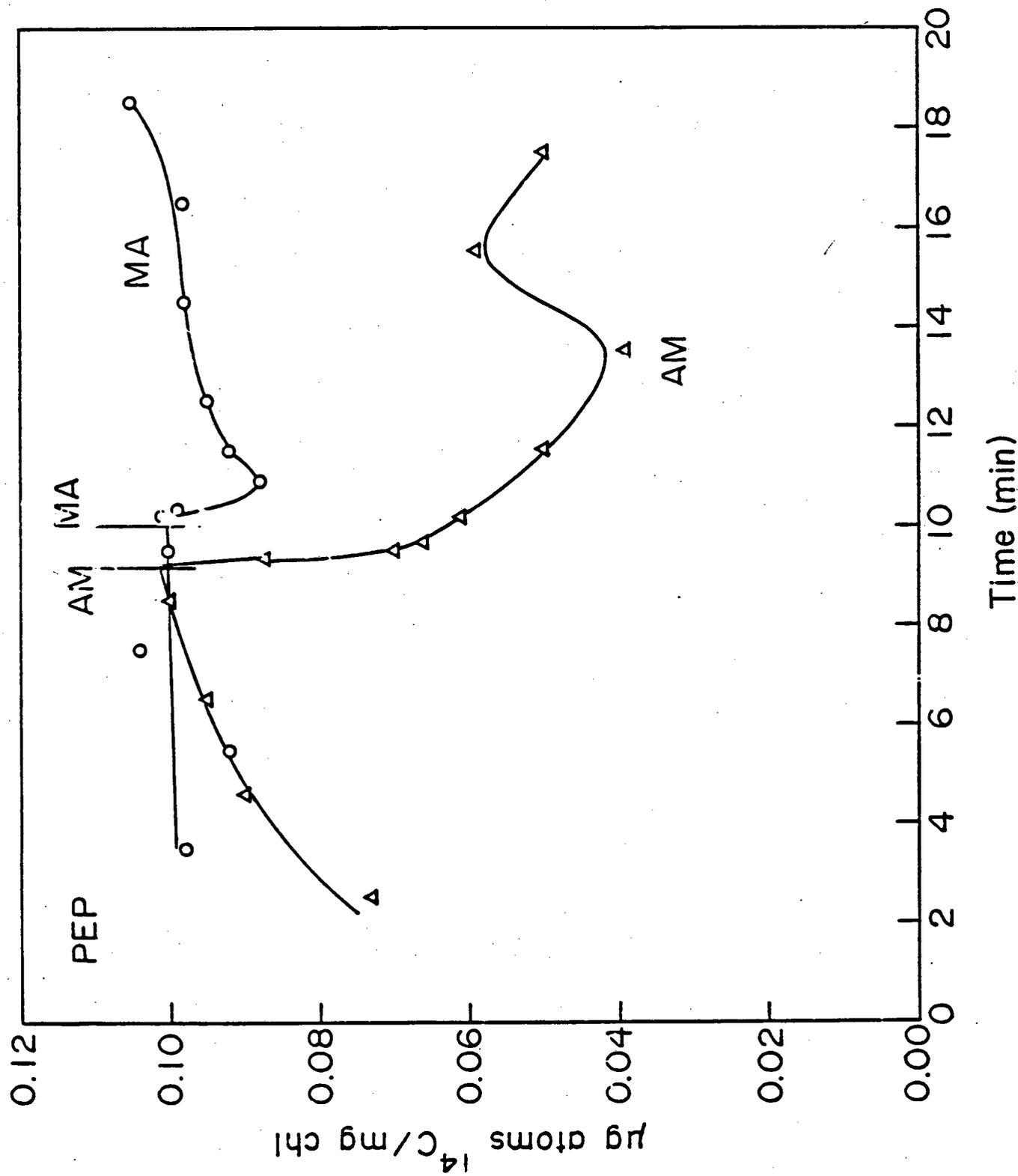
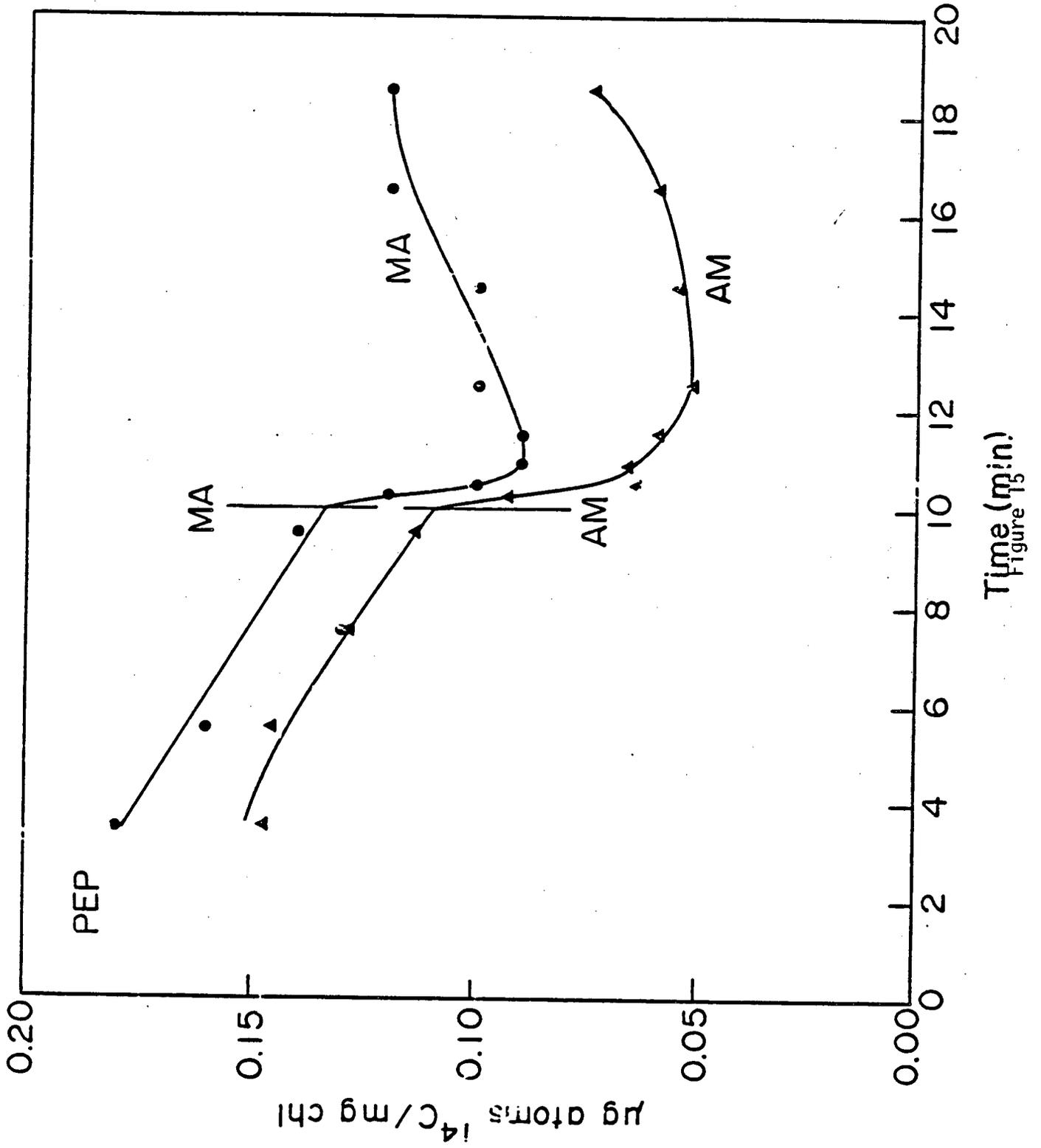


Figure 13

Fig. 13



Time (min)
Figure 14



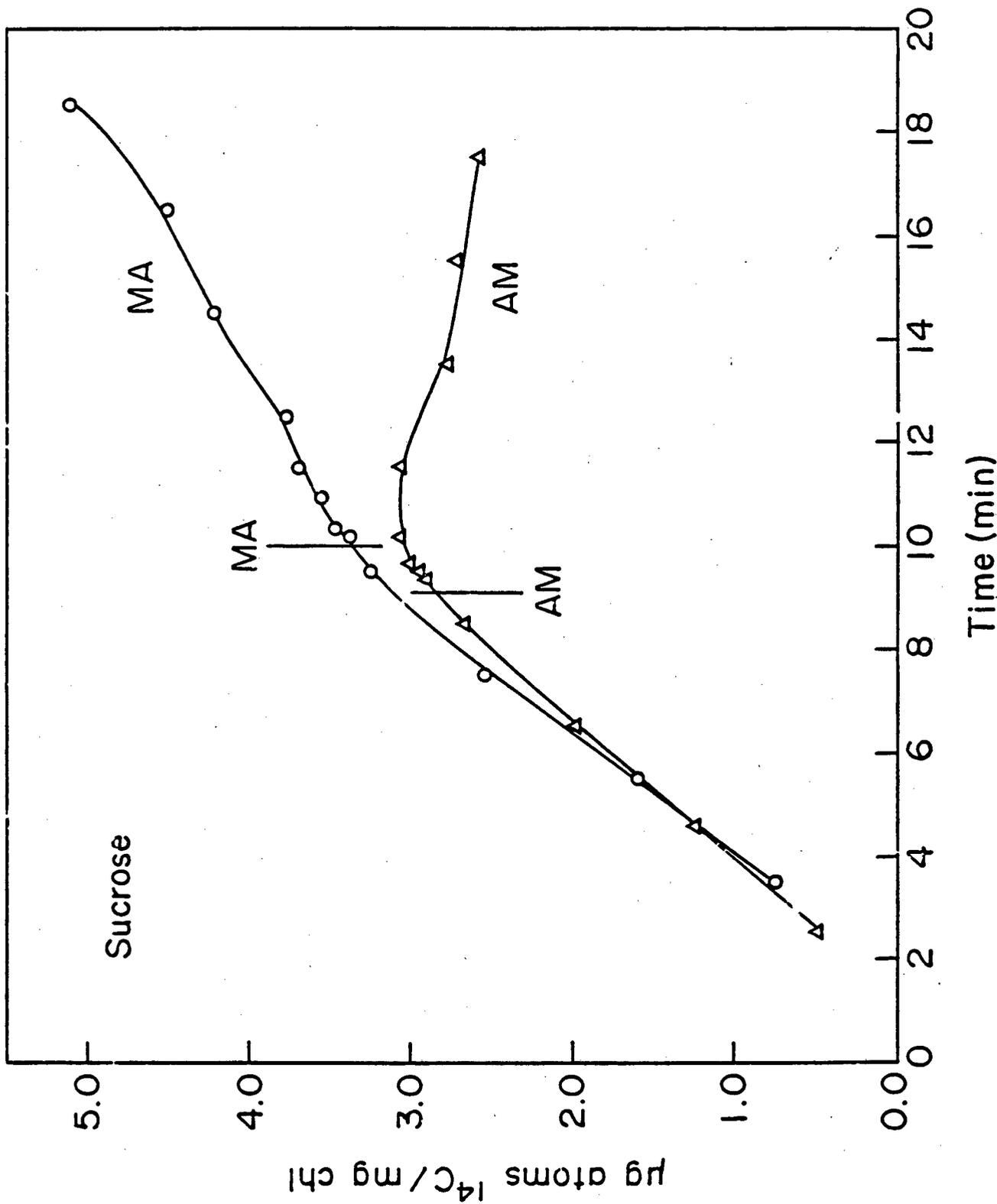


Figure 16

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