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Ernest O. Lawrence  
Radiation Laboratory

THE ACTION OF PHENETHYL ALCOHOL ON THE  
SYNTHESIS OF MACROMOLECULES  
IN ESCHERICHIA COLI

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THE ACTION OF PHENETHYL ALCOHOL ON THE  
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C. Prevost and V. Moses

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The Action of Phenethyl Alcohol on the Synthesis of Macromolecules  
in  
Escherichia coli

C. PREVOST and V. MOSES

Lawrence Radiation Laboratory, University of California, Berkeley,  
California

ABSTRACT

PREVOST, C. (Lawrence Radiation Laboratory, University of California, Berkeley, Calif.) AND V. MOSES. The action of phenethyl alcohol on the synthesis of macromolecules in Escherichia coli. J. Bacteriol. (1966). A kinetic study of the effects of various concentrations of phenethyl alcohol on the synthesis of RNA, DNA, protein and  $\beta$ -galactosidase in E. coli has confirmed that RNA synthesis, rather than DNA synthesis, is first and most affected by phenethyl alcohol. The presence of inducer did not protect  $\beta$ -galactosidase synthesis from inhibition by phenethyl alcohol. Little preferential inhibition of  $\beta$ -galactosidase synthesis was observed; this is in contrast to the severe catabolite repression which results from partial inhibition of total protein synthesis caused by chloramphenicol or starvation for a required amino acid. We found no evidence that messenger RNA synthesis was inhibited to a greater extent than total RNA synthesis.

The bacteriostatic action of phenethyl alcohol (6) was first ascribed in Escherichia coli to a specific and reversible effect on bacterial DNA synthesis (1). Sporulation and germination of Bacillus megaterium, however, were inhibited by phenethyl alcohol at a concentration which did not inhibit DNA synthesis (15). Although phenethyl alcohol inhibited the growth of DNA phages (4), it was also inhibitory for the growth of RNA phages (11). This suggested that the primary site of action may not necessarily be the inhibition of DNA synthesis. A recent paper has indicated that a lower concentration of phenethyl alcohol is required to give 50% inhibition of the incorporation of radiophosphorus into RNA than into DNA (14). On the basis of a preferential inhibition of the synthesis of ~~alkaline phosphatase~~ alkaline phosphatase compared with that of total protein, and some measurements of "mRNA" of induced  $\beta$ -galactosidase, Rosenkranz, Carr and Rose (13) suggested that the synthesis of messenger RNA was the principal point of attack by phenethyl alcohol.

We have measured the rates of protein, RNA and DNA synthesis in E. coli before and after the addition of various concentrations of phenethyl alcohol, and indeed find that RNA synthesis, but not necessarily messenger RNA synthesis, is the process showing greatest inhibition.

#### MATERIALS AND METHODS

Bacterium. E. coli Cavalli, a derivative of E. coli K-12, was obtained from Aleen Simmons of the Molecular Biology Department, University of California, Berkeley. It is auxotrophic for methionine and thymine.

Medium and growth. The bacteria were grown aerobically with stirring at 37°C in M-63 medium (12) supplied with 0.2% glycerol, 50 µg/ml methionine and 2.0 µg/ml thymine. In experiments where uracil incorporation was measured, the cells were usually grown in the presence of 70 µg/ml uracil; the cells preferentially used the externally added uracil (8). Growth was followed by measuring turbidity at 650 mµ in a Beckman DK-2 spectrophotometer. The doubling time was about 60 minutes.

Incorporation of labeled precursors. Labeled precursors (New England Nuclear Corp., Boston, Mass.) of the following specific radioactivities were added to the cultures at the beginning of the experimental period to measure the rates of labeling of macromolecules: methyl-H<sup>3</sup>-thymine, 300 µC/µmole; uracil-2-C<sup>14</sup>, 1.4 µC/µmole, methyl-C<sup>14</sup>-methionine, 1.4 µC/µmole. Samples (0.25 ml) from the culture of growing cells were added to 0.25 ml of 10% trichloroacetic acid (TCA) and left at 0° for at least 30 minutes. The whole 0.5 ml sample was transferred to a prewetted millipore filter (HAWP 025 00, 0.45 µ) and washed with 10 x 1 ml distilled water. The millipore filter was dissolved in 18 ml of scintillation fluid (3). The TCA insoluble fraction includes proteins, DNA and RNA. It was ascertained by two-dimensional paper chromatography of the acid hydrolysate of the TCA precipitate that the precursors were incorporated into the macromolecules without metabolic alteration. C<sup>14</sup> and H<sup>3</sup> were counted simultaneously in the Packard Tri-Carb scintillation counter.

Enzyme Induction and Assay. Isopropyl-thio- $\beta$ -D-galactopyranoside (IPTG), a gratuitous inducer of the lactose operon, was added to the bacterial cultures at a final concentration of  $5 \times 10^{-4}$  M. Galactosidase activity was assayed by measuring the rate of hydrolysis of o-nitrophenyl- $\beta$ -D-galactopyranoside as described by Kepes (2). One unit of enzyme activity is defined as that which catalyzes the hydrolysis of 1  $\mu$ mole substrate per minute per ml at 37 C, pH 7.4.

Protein. Total protein was measured chemically (1) and by following the incorporation of methionine- $C^{14}$ ..

## RESULTS

Effect on Turbidity, and the Synthesis of Protein and Induced  $\beta$ -galactosidase. The time course of the inhibitory effect on the synthesis of induced  $\beta$ -galactosidase by increasing concentrations of phenethyl alcohol is illustrated in Fig. 1. The differential rate of  $\beta$ -galactosidase synthesis ( $\beta$ -galactosidase versus methionine- $C^{14}$  incorporation) is definitely not depressed at lower concentrations of phenethyl alcohol but is even slightly stimulated (Fig. 2A). Higher concentrations of phenethyl alcohol cause some degree of preferential inhibition which increases with time (Fig. 2B, 2C).

Phenethyl alcohol, at a concentration of 0.30% was sufficient to cause an almost immediate cessation of protein synthesis and increase in turbidity (Fig. 3). At a slightly lower concentration (0.28%) protein synthesis was not entirely inhibited and the following kinetic phenomenon occurred: protein and induced  $\beta$ -galactosidase synthesis were sharply inhibited for a period of 20 to 40 minutes, then recovered somewhat during the next 2 hours before being completely inhibited thereafter (Fig. 4). At no time, at these high concentrations of phenethyl alcohol, was there any significant uncoupling between general protein synthesis and induced  $\beta$ -galactosidase synthesis. Furthermore,  $\beta$ -galactosidase synthesis, induced at various times after the addition of phenethyl alcohol, followed the same pattern of behavior (Fig. 5) as  $\beta$ -galactosidase synthesis induced 20 minutes

before the addition of phenethyl alcohol. The rate of  $\beta$ -galactosidase synthesis was inhibited to the greatest extent 10 minutes after the addition of phenethyl alcohol and to the least extent 60 minutes later, during the recovery period. In an experiment similar to the one reported in Figure 5 IPTG was added 3 hours after phenethyl alcohol (0.30%) and although protein synthesis was inhibited by about 98%, the differential rate of  $\beta$ -galactosidase synthesis was not reduced by more than 25%.

Effect on Nucleic Acid Synthesis. DNA synthesis was inhibited after one hour by about 0.30% phenethyl alcohol (Fig. 6). The small extent of incorporation of thymine- $H^3$  added 5 hours after 0.30% phenethyl alcohol revealed that no major portion of the DNA was turning over at that time. The synthesis of DNA and RNA were compared by simultaneously adding thymine- $H^3$ , uracil- $C^{14}$  and phenethyl alcohol (0.30%) (Fig. 7). DNA synthesis continued at an undiminished rate for at least 1 hour before slowing down. The amount of DNA made during that period corresponded to 40-60% of the amount present at the time of addition of phenethyl alcohol. The incorporation of uracil was relatively low. The rate of uracil incorporation dropped by at least 90% within 20 minutes of the addition of phenethyl alcohol (Fig. 8). The cells were nevertheless able to incorporate uracil during a 2 minute pulse at a low rate for at least 3 hours. Continuous measurement of the incorporation of uracil- $C^{14}$  added 30 minutes before

phenethyl alcohol showed that not only was uracil incorporation inhibited within a few minutes but that RNA also underwent a progressive breakdown (Fig. 9B). Similar measurements at 0.25% phenethyl alcohol again show that RNA synthesis was more inhibited than DNA synthesis (Fig. 9A). Following an initial and complete inhibition, which lasted approximately 20 minutes, RNA synthesis recovered to only 10% of the rate immediately preceding the addition of phenethyl alcohol. By comparison the rate of DNA synthesis was slowed down by about only 40% during the first hour and the rate of protein synthesis was even less affected.

## DISCUSSION

Phenethyl alcohol has been widely used as a specific inhibitor of nucleic acid synthesis. There is no general agreement, however, as to the actual site of action or even on the relative extent of inhibition of the biosynthesis of the various macromolecules.

Treick and Konetzka (18) have followed the kinetics of the inhibition of DNA synthesis as well as that of RNA synthesis by measuring the incorporation of labelled thymine and uracil after the addition of phenethyl alcohol to the culture medium. We have obtained similar kinetic results as far as the inhibition of DNA synthesis is concerned, but have been unable to confirm the response of RNA synthesis to phenethyl alcohol which they observed. In their experiments uracil incorporation continued unimpaired for up to 4 hours in the presence of 0.32% phenethyl alcohol. They do not indicate, however, the rate of uracil- $C^{14}$  incorporation in the absence of phenethyl alcohol. In our experiments, we found that this concentration of phenethyl alcohol not only stopped total RNA synthesis, but breakdown of the previously formed RNA occurred (Fig. 9B). The rate of uracil uptake with phenethyl alcohol reported by Treick and Konetzka (18) may have been a very small fraction of the rate at which uracil was taken up prior to the addition of the inhibitor. If this were the case, their findings would agree with the observations reported in this communication (Figs. 7 and 8). Our results concerning the inhibition of RNA synthesis are in agreement with those of Rosenkrantz et al. (14).

Maaløe and Hanawalt (7) found that inhibition of the synthesis

of RNA and protein by the removal of uracil and arginine from E. coli 15 T<sup>-</sup>U<sup>-</sup>A<sup>-</sup>, which requires these substances for growth, prevented the initiation of a new cycle of DNA replication while permitting the completion of the cycle then in progress. In view of this observation it is to be expected that phenethyl alcohol, which caused a severe inhibition of RNA and protein synthesis, would ultimately have a similar effect on the synthesis of DNA.

Rosenkranz et al. (19) have demonstrated that low concentrations of phenethyl alcohol inhibit the synthesis of alkaline phosphatase to a much greater extent than the synthesis of proteins in general. A study by them of the effects of phenethyl alcohol on the induction of  $\beta$ -galactosidase seemed to indicate that, at levels which were not inhibitory to other processes, phenethyl alcohol interfered with the function or biosynthesis of  $\beta$ -galactosidase-specific messenger RNA. They have therefore proposed that phenethyl alcohol selectively inhibits the synthesis of messenger RNA. This explanation, however, is not consistent with the results reported in the present communication.

It has been estimated that 3-4% of all the RNA in E. coli has a messenger function (5). If all messenger RNA is labile, then a total inhibition of messenger RNA synthesis, which would rapidly be reflected in a total cessation of protein synthesis, would appear as only a 4% inhibition of total RNA synthesis. However, in experiments reported above the partial reduction in protein synthesis has always been associated with a very much greater inhibition of RNA synthesis than 4%. Were messenger RNA's for the bulk of the cellular protein

not short-lived (9), complete inhibition of messenger RNA synthesis would not immediately have affected the synthesis of the total proteins. Messenger RNA for  $\beta$ -galactosidase, however, is known to be very labile (2) so that we would expect a rapid preferential inhibition of  $\beta$ -galactosidase synthesis in the presence of phenethyl alcohol; such preferential inhibition has not been observed.

In contrast to the earlier findings with alkaline phosphatase and  $\beta$ -galactosidase (13), we found only a comparatively slight preferential inhibition of  $\beta$ -galactosidase synthesis (Fig. 2). A comparison of our findings with those of Rosenkrantz et al. (13) is shown in Table 1. Thus, if the large inhibitory effects on  $\beta$ -galactosidase synthesis, observed by Rosenkrantz et al. in the first few minutes after phenethyl alcohol addition, were due, as they suggest to preferential inhibition of the specific messenger RNA, such an inhibition must be a very transitory phenomenon from which the cells recovered rapidly (see Fig. 1).

The presence of inducer did not protect  $\beta$ -galactosidase synthesis from phenethyl alcohol. Induction of  $\beta$ -galactosidase synthesis at various times after the addition of phenethyl alcohol (Fig. 5) followed the same pattern of inhibition and recovery as  $\beta$ -galactosidase synthesis induced before the addition of phenethyl alcohol (Fig. 4).

While our kinetic experiments do not demonstrate that phenethyl alcohol is more inhibitory for the synthesis of messenger RNA than for other types of RNA, it is nevertheless obvious that a very great inhibition of RNA synthesis in general must eventually result in a lack of ribosomal and transfer RNA necessary for the translation of

messenger RNA, and consequently cause a decrease in the rate of protein synthesis. We have no immediate explanation for the period of preferential synthesis of  $\beta$ -galactosidase at low concentrations of phenethyl alcohol (Fig. 2A).

The small degree of catabolite repression with phenethyl alcohol is rather unusual. It has frequently been observed in the past that when protein synthesis is partially inhibited, the differential rate of  $\beta$ -galactosidase synthesis falls as a consequence of catabolite repression. For instance, Sypherd, Strauss and Traffers (17) found that when protein synthesis was inhibited by 40-50% with chloramphenicol the differential rate of  $\beta$ -galactosidase synthesis fell by 68%. Nakada and Magasanik (10) have noted that "cells cannot be induced in a medium containing glycerol as well as chloramphenicol; as in all cases of inhibition of protein synthesis without the removal of the source of catabolites, this effect may be ascribed to catabolite repression". Inhibition by phenethyl alcohol does not seem to follow such a rule, since, in the presence of glycerol, inhibition of protein synthesis by as much as 98% only resulted in a 25% reduction of the differential rate of induced  $\beta$ -galactosidase synthesis. We might even conclude from this that one of the modes of action of phenethyl alcohol is interference with the operation of catabolite repression.

#### ACKNOWLEDGMENTS

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TABLE 1. Comparison of the inhibition of alkaline phosphatase,  $\beta$ -galactosidase, and protein increases after one hour with phenethyl alcohol at 37 C.

Phenethyl alcohol concentration (v/v)	0.10%	0.15%	0.20%	0.25%
<u>Rosenkranz et al.*</u>				
Protein (turbidity) (% of control)	73	-	57	-
Alkaline phosphatase (% of control)	5	-	0	-
Alkaline phosphatase/protein	0.07	-	0	-
<u>Present results</u>				
Protein** (% of control)	71	61	-	22
$\beta$ -galactosidase (% of control)	87	45	-	16
$\beta$ -galactosidase/protein	1.23	0.74	-	0.73

\*The values were computed from the results presented in Figure 1 from Rosenkranz, Carr and Rose (13).

\*\*These results were calculated from the experiment described in Figures 1 and 2. The estimate of protein synthesis was the same whether turbidity or methionine- $C^{14}$  incorporation was used.

LIST OF FIGURES

Figure 1 The effect of phenethyl alcohol on the synthesis of induced  $\beta$ -galactosidase. IPTG was added at time zero to four parallel cultures of exponentially growing cells. Phenethyl alcohol was added 32 minutes later:  $\Delta$ , 0% (v/v);  $\circ$ , 0.10%;  $\ominus$ , 0.15%;  $\triangle$ , 0.25%.

Figure 2 The effect of phenethyl alcohol on the rate of induced  $\beta$ -galactosidase synthesis versus protein synthesis as measured by methionine- $C^{14}$  incorporation. Same experiment as in Fig. 1. Phenethyl alcohol was added at the arrow: A  $\circ$  control,  $\ominus$  0.10%; B  $\circ$  control,  $\ominus$  0.15%; C  $\circ$  control,  $\ominus$  0.25%.

Figure 3 Effect of phenethyl alcohol (0.30%) on turbidity and total protein.

Figure 4 Effect of phenethyl alcohol (0.28%) on total protein and induced  $\beta$ -galactosidase synthesis.

Figure 5 Inducibility of  $\beta$ -galactosidase after the addition of phenethyl alcohol (0.28%). Phenethyl alcohol was added to a culture of exponentially growing cells. At various times samples of the culture were removed and  $\beta$ -galactosidase synthesis induced with IPTG.  $\beta$ -Galactosidase activity was then followed for the next 25 min. Induction started at the following times in relation to addition of phenethyl

alcohol:  $\Delta$ , minus 20 min.;  $\circ$ , plus 10 min.;  $\ominus$ , plus 70 min.;  
 $\square$ , plus 130 min.;  $\triangle$ , plus 190 min.

Figure 6 Effect of phenethyl alcohol (0.30%) on thymine incorporation in exponentially growing cells. Culture A was grown in presence of thymine- $H^3$ . Culture B was grown in a parallel flask but thymine- $H^3$  was not added until 5 hours after the addition of phenethyl alcohol.

Figure 7 Effect of phenethyl alcohol (0.30%) on thymine and uracil incorporation. Thymine- $H^3$  and uracil-2- $C^{14}$  added simultaneously with phenethyl alcohol to exponentially growing cells.

Figure 8 Effect of phenethyl alcohol (0.30%) on the rate of labeling of RNA with uracil-2- $C^{14}$ . The cells were grown in the absence of uracil. At various intervals 0.25 ml samples were added to 0.025 ml of uracil-2- $C^{14}$  (15.8  $\mu$ moles; 31.6  $\mu$ C/ $\mu$ mole), incubated for 2 min. at 37 C and killed by the addition of 0.25 ml of 10% trichloroacetic acid at 0 C.

Figure 9 Effect of phenethyl alcohol on thymine- $H^3$  and uracil-2- $C^{14}$  incorporation in exponentially growing cells. A, 0.25% phenethyl alcohol; B, 0.30% phenethyl alcohol.

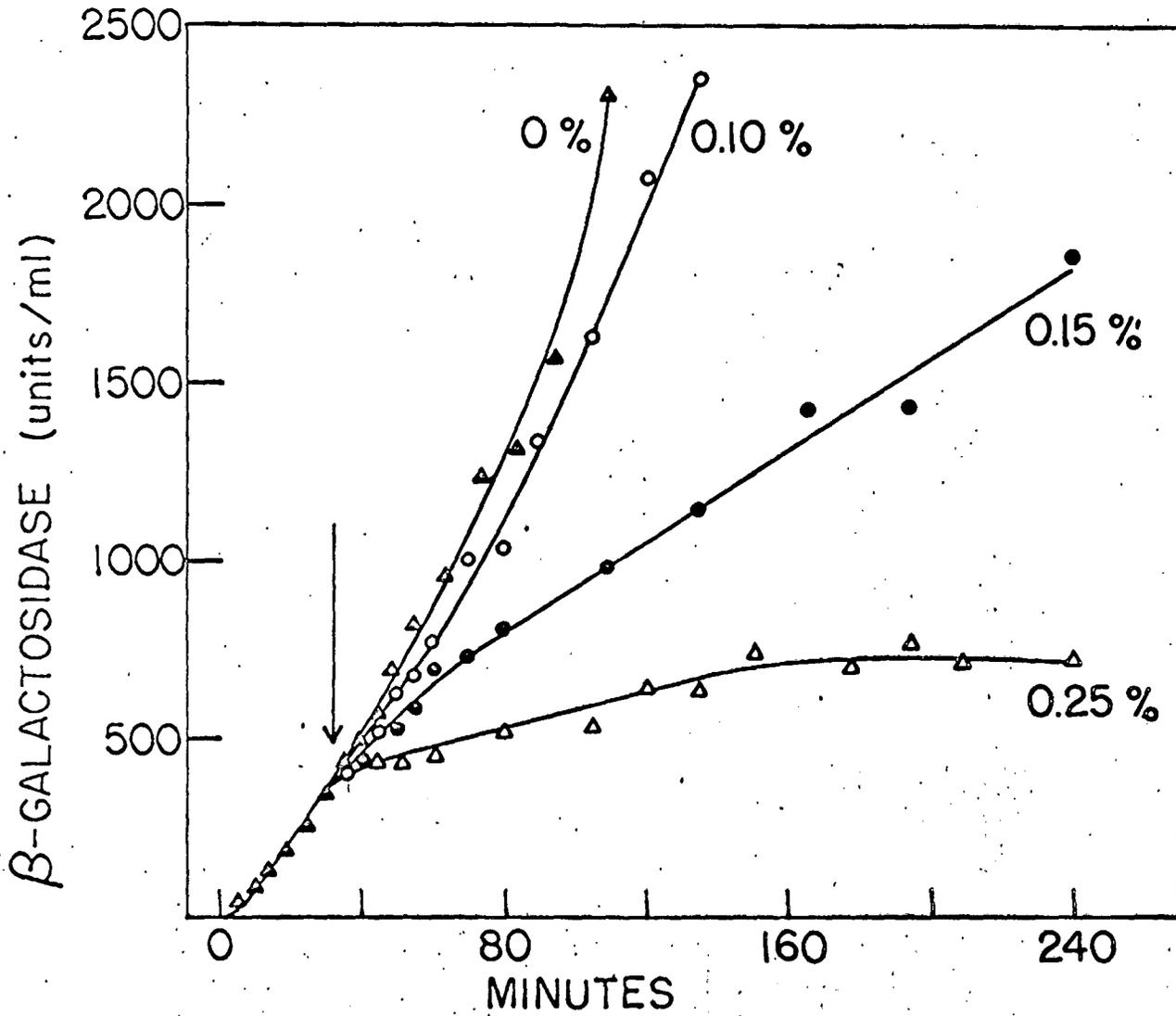
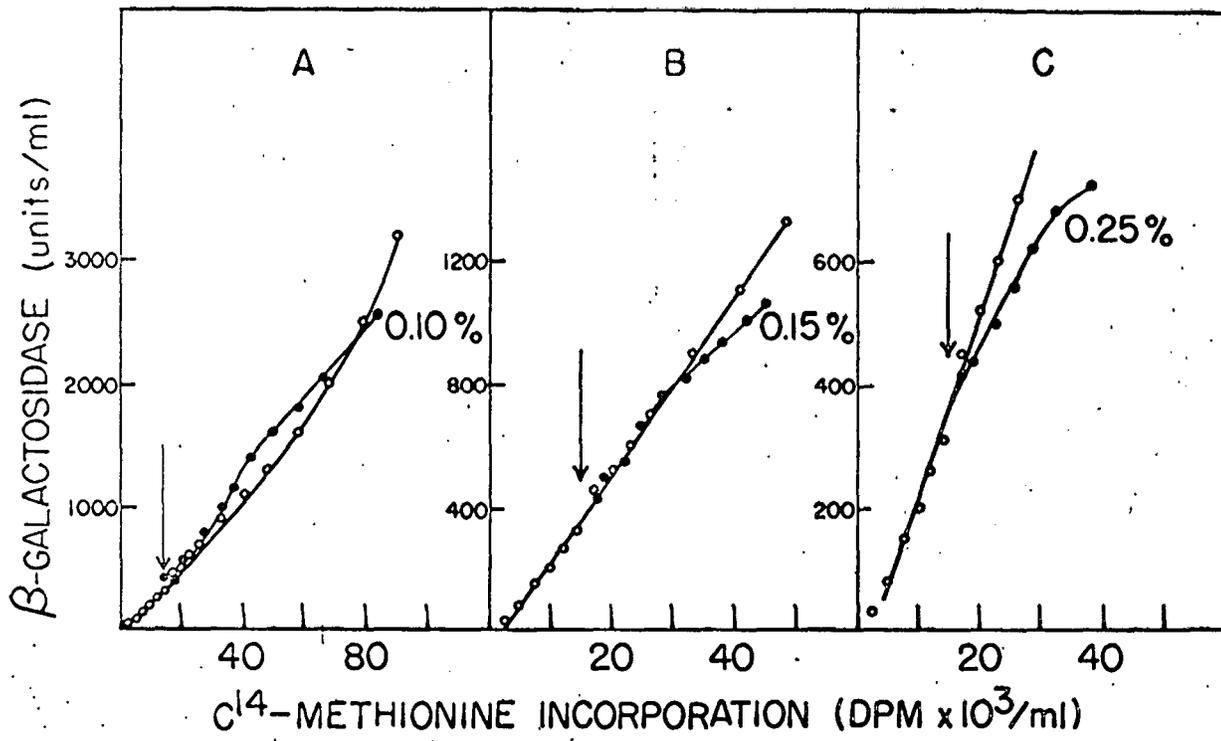


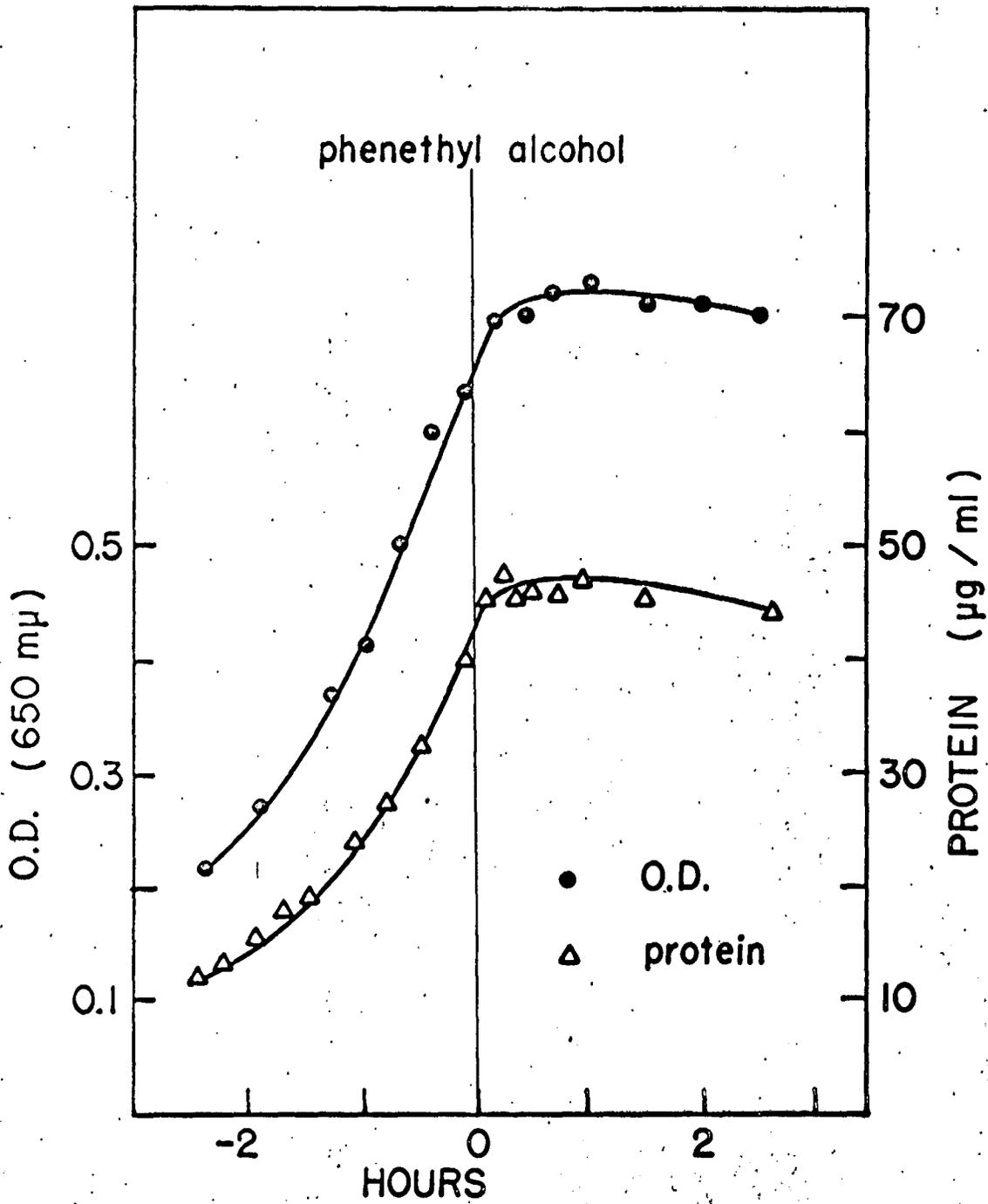
Fig. 1

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Fig. 2



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Fig. 3

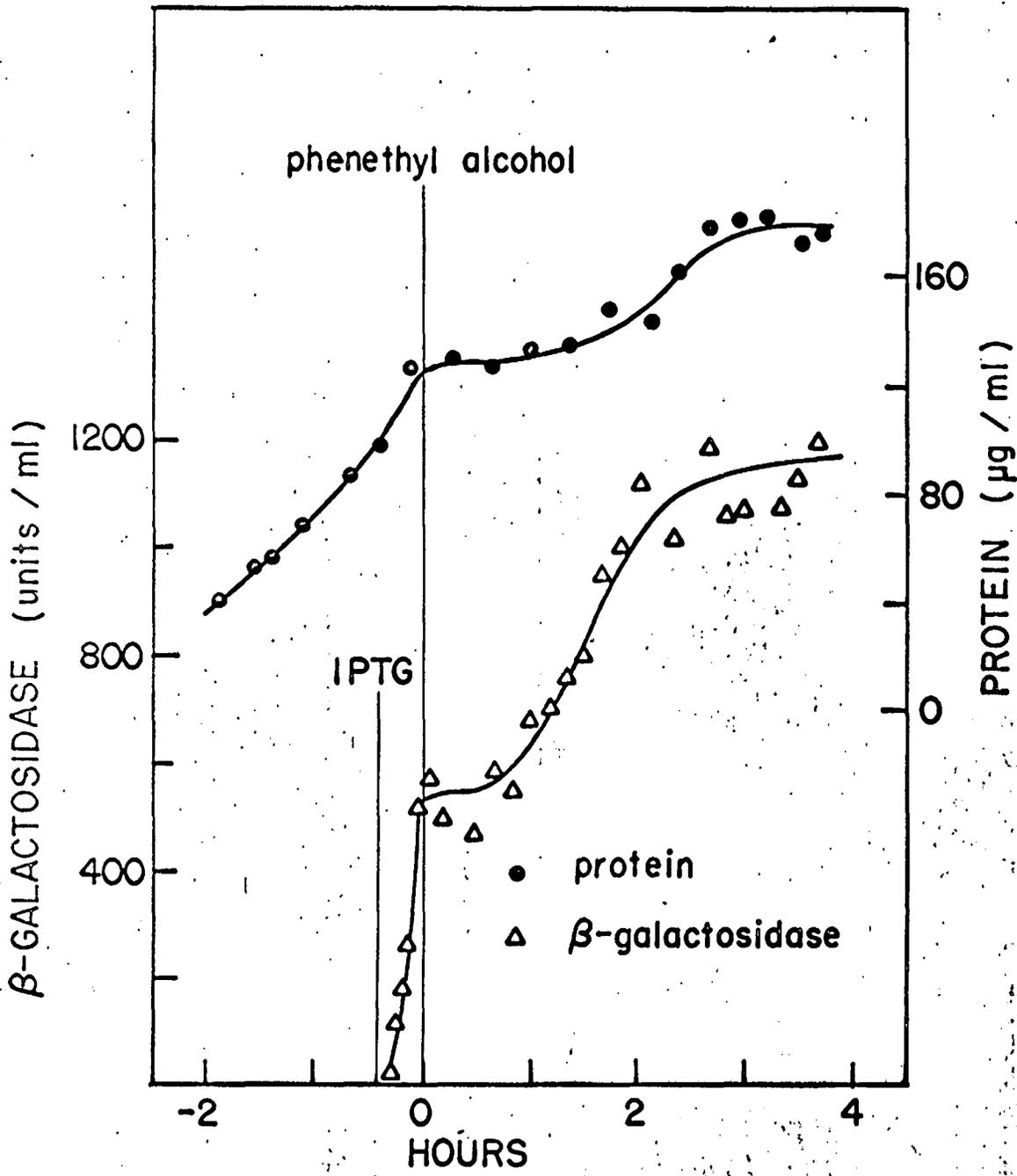


Fig. 4

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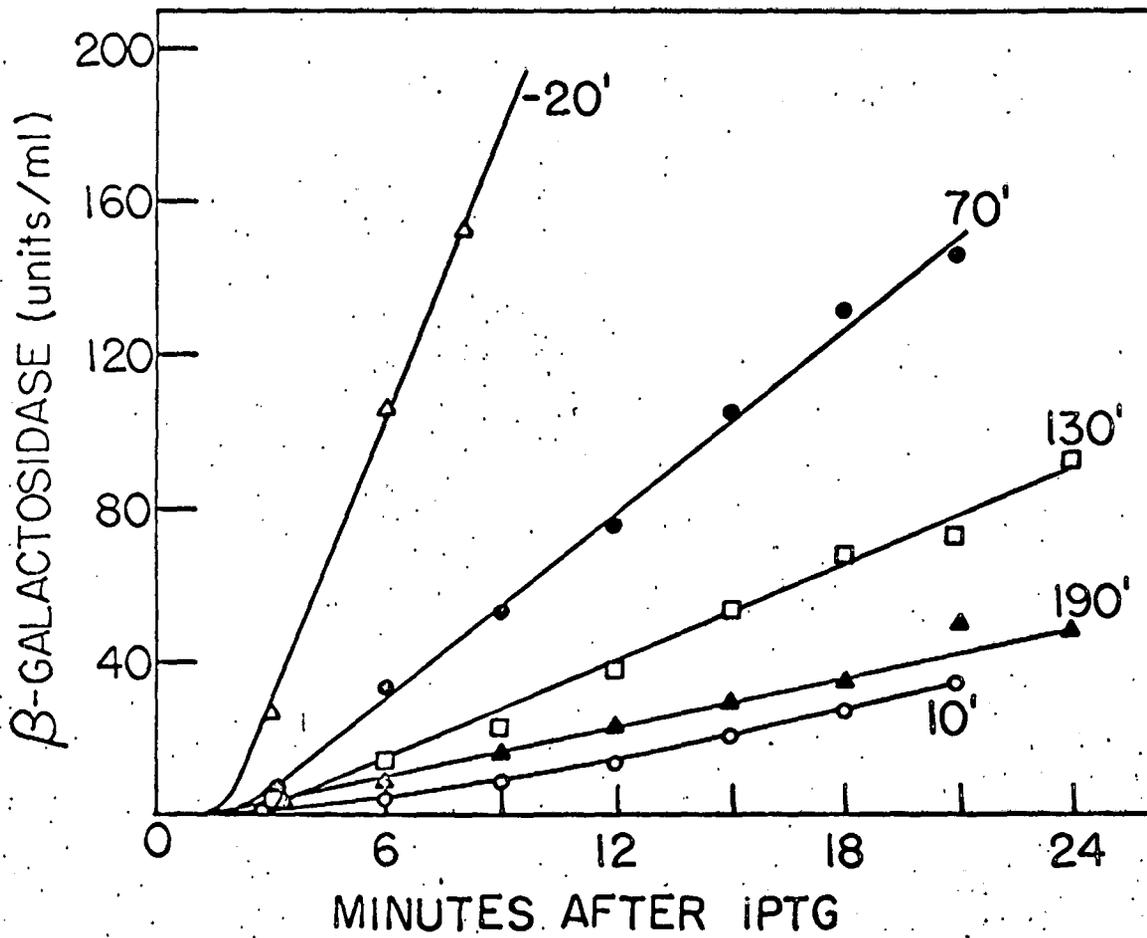
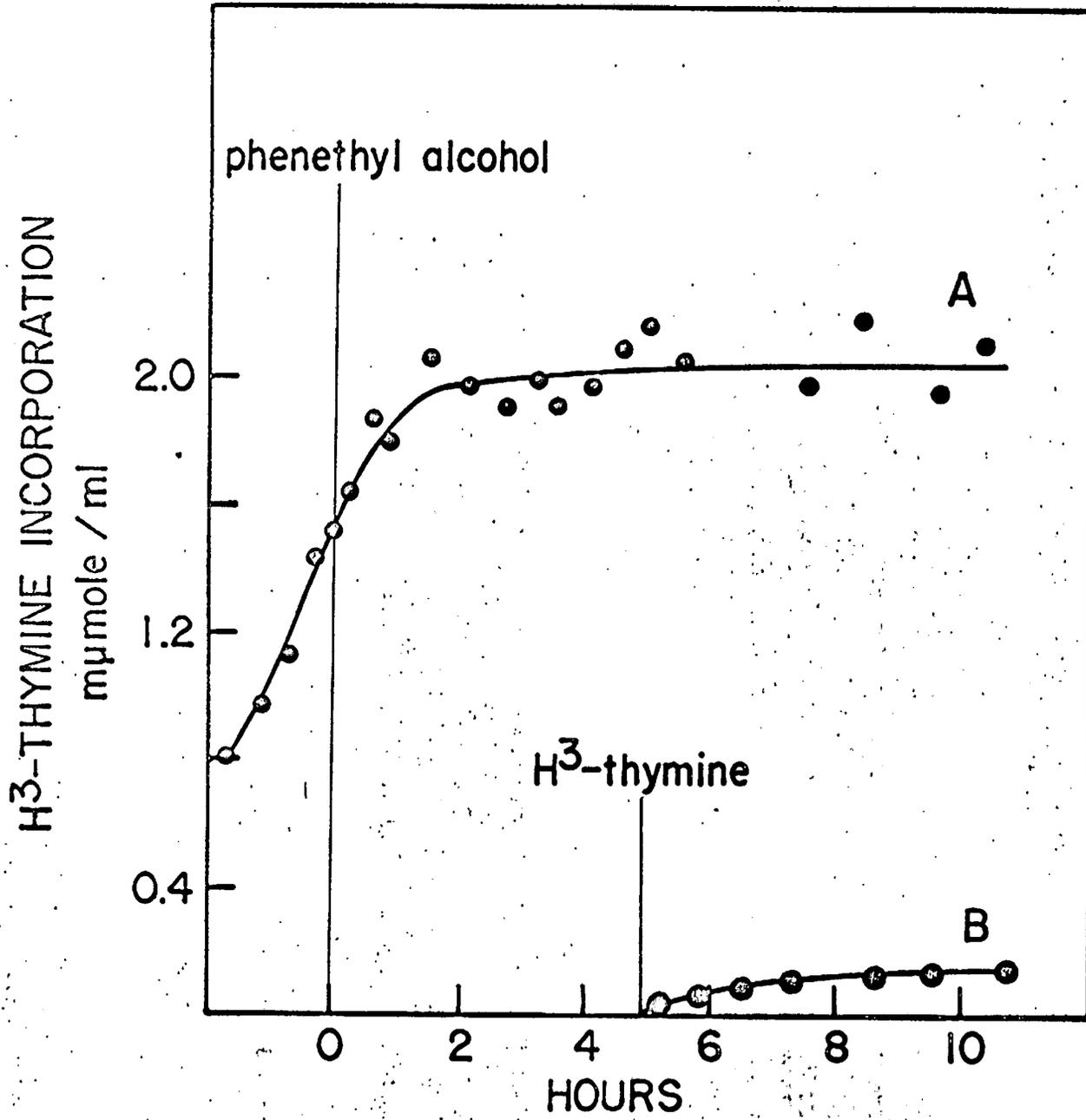


Fig. 5

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Fig. 6

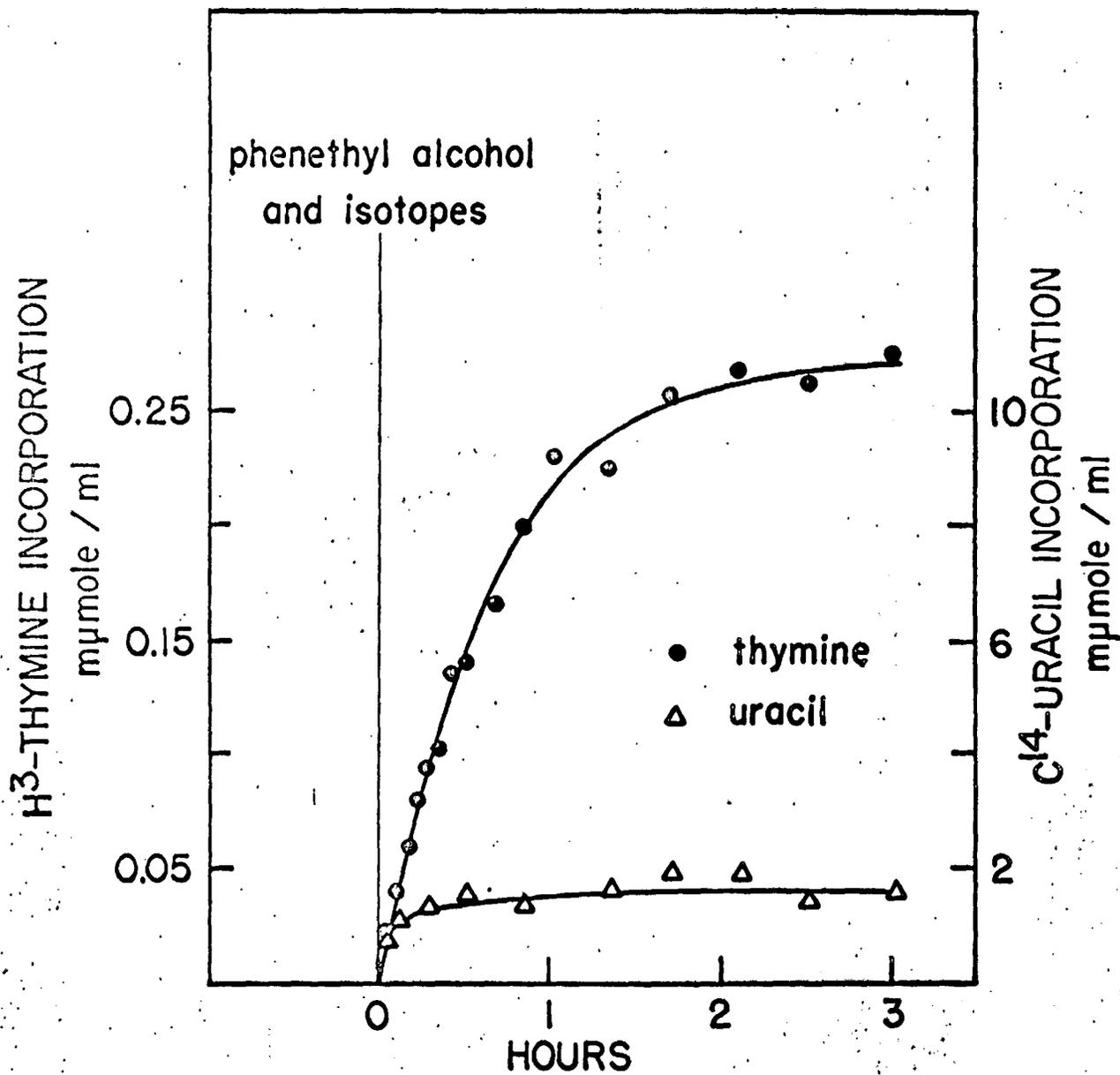


Fig. 7

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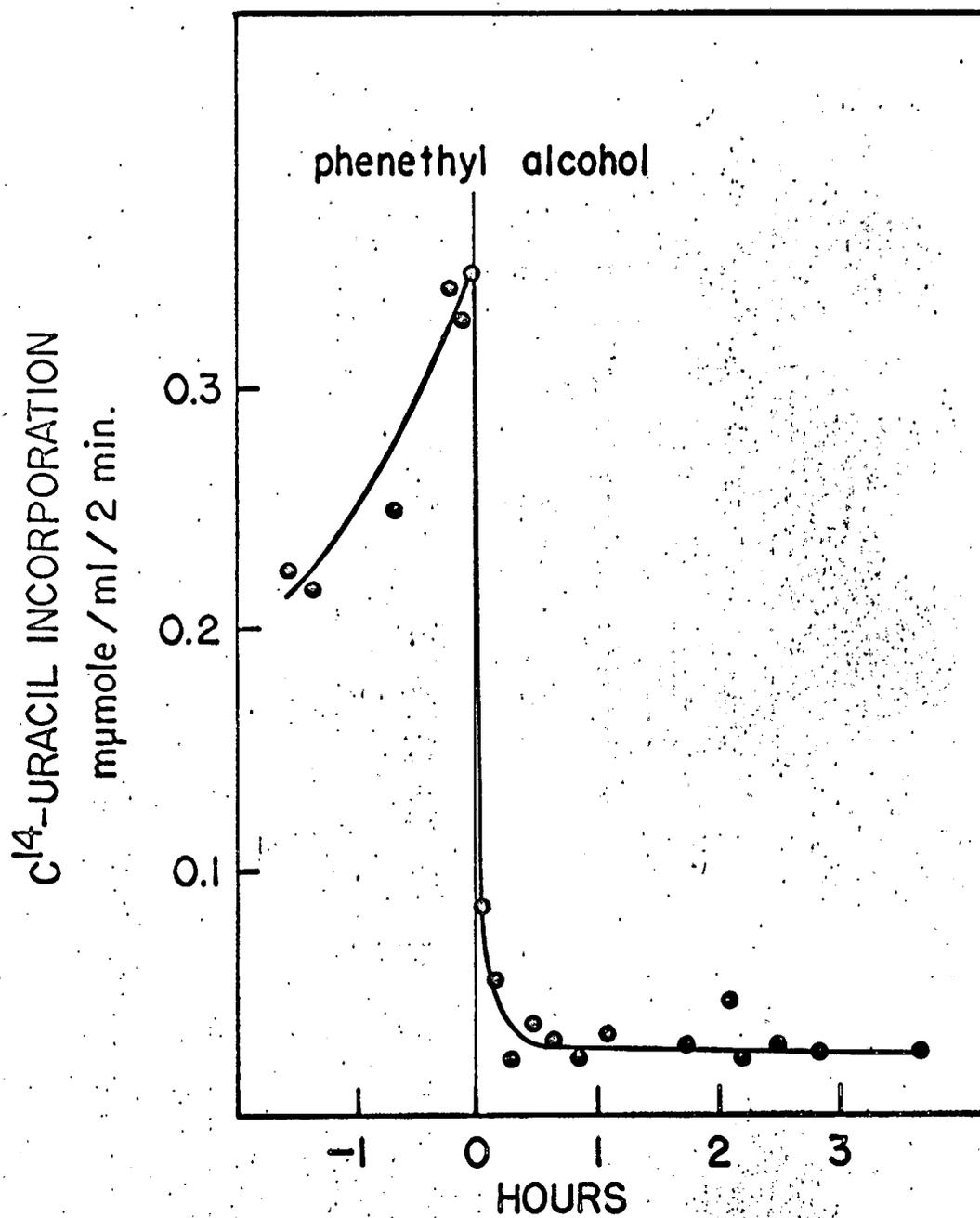


Fig. 8

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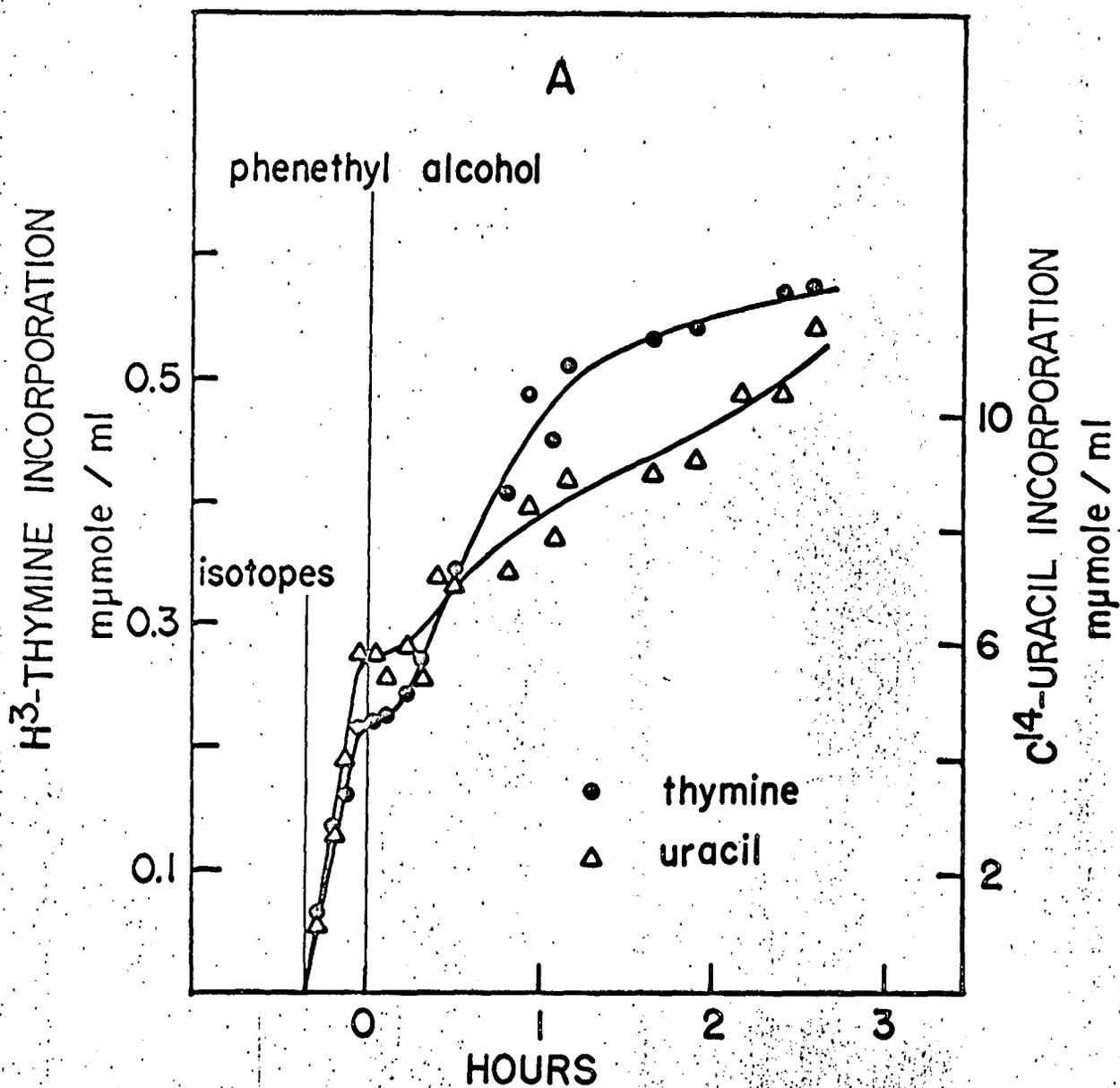


Fig. 9(A)

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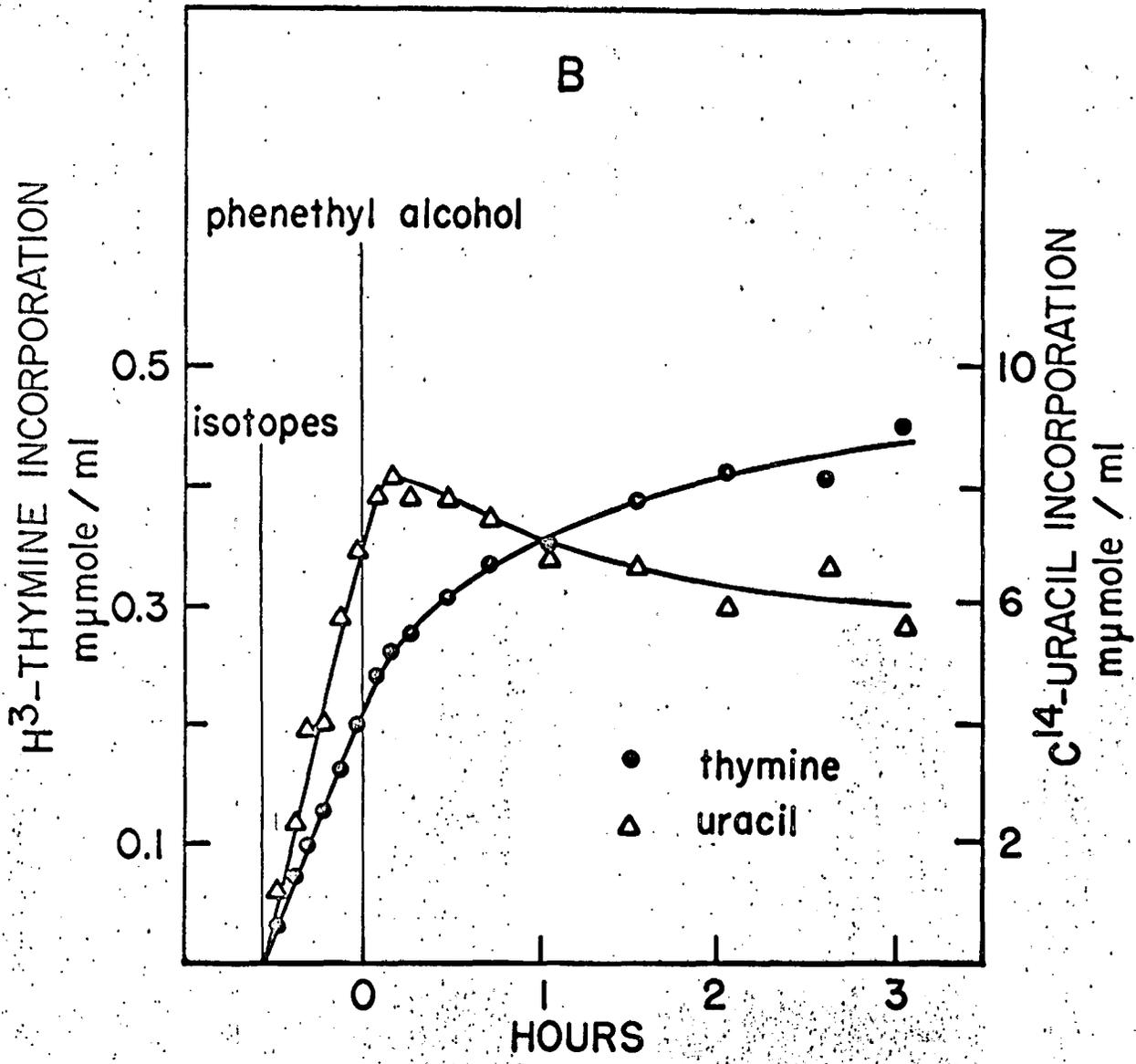


Fig. 9(B)

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