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Azotobacter vinelandii RNA Polymerase.

II. Effect of Ribonuclease on Polymerase Activity

Joseph S. Krakow

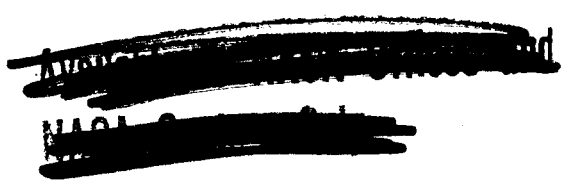
Space Sciences Laboratory
University of California
Berkeley, California

INTRODUCTION

RNA synthesis by RNA polymerase directed by a native DNA template evinces a characteristic and unusual time course for an enzymatic reaction. The synthesis is initially rapid and linear, then subsequently slows, and a plateau is established. These kinetics are characteristic of the enzyme as isolated either from Azotobacter vinelandii (1), E. coli (2,3), or M. lysodeikticus (4), are noted at all stages of purification (5), and are independent of the amount of polymerase added to the incubations (1). It has been demonstrated that a variety of polyribonucleotides can inhibit the DNA-directed RNA polymerase (4,6,7,8). From such findings and from a consideration of the nature of the kinetics it has been a general conclusion that RNA polymerase is inhibited by RNA produced during the course of the reaction. This paper will seek to demonstrate the validity of this contention.

EXPERIMENTAL PROCEDURE

Unlabeled ribonucleoside polyphosphates were obtained from P-L Laboratories, Inc., Milwaukee, Wisconsin. ATP-8-^{14}C and GTP-8-^{14}C were purchased from Schwarz BioResearch, Inc., Orangeburg, New York. Calf thymus DNA and pancreatic RNase were products of Worthington Biochemical Corporation, Freehold, New Jersey. The RNase (0.5 mg/ml in .01 M Tris, pH 7.2) was heated for 10 minutes at about 90°C to denature any contaminating DNase (9). Spermidine was obtained from Calbiochem, Inc., Los Angeles, California and Sephadex G200 from Pharmacia Fine Chemicals, Inc., New Market, New Jersey. T1 RNase was a product of Sankyo Ltd., Tokyo, Japan, and was generously donated by Dr. Mituru Takanami.



RNA polymerase from A. vinelandii (strain OP) was prepared by a modification of the published procedure (10). The hydroxylapatite fraction was further treated by gel filtration on a Sephadex G200 column (2.5 cm x 30 cm.) equilibrated with 0.02 M potassium phosphate buffer, pH 6.8 containing 0.2 M KCl and 0.001 M EDTA. The enzyme appeared after passage of about 50 ml of buffer and the most active fractions were pooled, brought to a concentration of 55% $(NH_4)_2SO_4$, and centrifuged at 15,000 g for 20 minutes. The enzyme was dissolved in 0.02 M potassium phosphate, pH 6.8 containing 0.001 M EDTA and stored at $-15^{o}C$ at a concentration of 2 mg/ml.

γ^{32}P-ATP (AMP-P-^{32}P) was synthesized by the procedure of Glynn and Chappell (11), which was coupled with yeast nucleoside diphosphokinase (12) to synthesize γ^{32}P-UTP and γ^{32}P-GTP. These were isolated after chromatography on Dow 1 Cl^- [1].

Nitrocellulose membrane filters (0.4μ pore size) were obtained from Schleicher and Schuell, Keene, New Hampshire. The filters were stored in 0.01 M ATP (adjusted to pH 6.0 with sodium pyrophosphate) in order to lower the blank values for the ^{14}C-ATP and ^{14}C-GTP polymerase assays. For assays measuring $^{32}PP_i$ release the filters were stored in 0.01 M sodium pyrophosphate, adjusted to pH 6.0 with KH_2PO_4.

Assay of RNA Polymerase

RNA synthesis was followed by determining the incorporation of ^{14}C-ATP or ^{14}C-GTP into acid-insoluble material retained on membrane filters. The reaction was stopped by addition of 0.1 ml of saturated sodium pyrophosphate followed by 2 ml of ice cold 5% TCA [2]. The precipitates were washed on the membrane filters with 4 x 5 ml of cold 5% TCA. The filters were dried, and placed on planchetts, and the radioactivity was determined.

$^{32}PP_i$ release from $\gamma^{32}P$-labelled ribonucleoside triphosphates was followed by determining the amount of ^{32}P which was rendered nonadsorbable to charcoal. The reaction was stopped by addition of 1 ml of 0.01 M sodium pyrophosphate adjusted to pH 6.0 with KH_2PO_4 and 1 ml of a 10% charcoal suspension (Pfanstiehl acid washed Norit in 0.01 M sodium pyrophosphate adjusted to pH 6.0 with KH_2PO_4). After stirring, the suspension was filtered through a membrane filter to remove the charcoal and 0.5 ml of the filtrate was plated, dried and the radioactivity was determined.

The incubation conditions used for ^{14}C-labeled nucleotide incorporation of $^{32}PP_i$ release were identical. The reaction mixture contained in 0.25 ml:80 mM Tris buffer, pH 8.2, 20 mM $MgSO_4$, 32 mM mercaptoethylamine, calf thymus DNA equivalent to 150 mμ moles of deoxyribonucleotides, 0.4 mM of the indicated labeled ribonucleoside triphosphate, 0.4 mM of each of other three ribonucleoside triphosphates and RNA polymerase as indicated. The reaction mixtures were incubated at 37°.

RESULTS

The results presented in Table 1 demonstrate the general requirements of Azotobacter RNA polymerase for release of $^{32}PP_i$ from $\gamma^{32}P$-labeled GTP. The omission of Mg^{++}, DNA, or RNA polymerase results in a virtual lack of $^{32}PP_i$ formation. When $\gamma^{32}P$-GTP is the sole nucleotide in the reaction mixture one notes that some $^{32}PP_i$ was formed, this probably is the result of a limited ability of the enzyme to form poly G. These requirements for polymerase activity assayed for PP_i release are consistent with those in which incorporation of ^{14}C-labeled nucleotides into RNA are used (10). However, when one adds RNase to reactions where incorporation of nucleotides into RNA is followed one finds that only about 1%-5% of the label is acid insoluble. In marked contrast to this are the results shown in Table 1 showing that $^{32}PP_i$ release is stimulated by the addition of RNase. $^{32}PP_i$ release requires the presence of RNA polymerase since pancreatic and T1 RNase by themselves are unable to effect $^{32}PP_i$ (or $^{32}P_i$) release. One may therefore assume that the $^{32}PP_i$ formed is a consequence of polymerase-catalyzed phosphodiester formation and not due to phosphatases or other enzymes contaminating the nuclease preparations.

The data presented in Table 2 further demonstrate that the $^{32}PP_i$, formed is

a consequence of RNA synthesis. Actinomycin D is known to be a potent and very specific inhibitor of the native DNA directed synthesis of RNA by polymerase (13,14). Furthermore all the effects of actinomycin have been ascribed to its ability to specifically bind to native DNA (15). If the $^{32}PP_i$ formed in the reaction was caused by some factor other than polymerase it should be possible to demonstrate PP_i (or P_i) release in the presence of actinomycin. The concentration of antibiotic used was sufficient to result in a 95% inhibition of RNA synthesis. As shown in Table 2 the release of $^{32}PP_i$ is very sensitive to inhibition by actinomycin both in the presence or absence of added nuclease. The data also show that the amount of $^{32}PP_i$ released from UMP-P-^{32}P relative to that released from GMP-P-^{32}P in the standard reaction or in the nuclease-stimulated reaction is in reasonable agreement with what would expect when calf thymus DNA (AT/GC=1.3) is the template (10). The findings indicate that RNA polymerase is transcribing RNA normally even though the RNA formed is rapidly degraded in the presence of the nucleases.

The addition of either pancreatic RNase or T1 RNase results in a marked stimulation of polymerase activity (Table 3). In order to obtain maximal stimulation of PP_i release both nucleases should be present. The requirement for both nucleases is probably due to a limited ability of the oligonucleotides resistant to T1 RNase or pancreatic RNase alone to interfere with polymerase activity.

The data indicate that PP_i release is a valid measure of polymerase activity and that the addition of pancreatic RNase and T1 RNase stimulates polymerase activity. This release of $^{32}PP_i$ from γ^{32}P-ribonucleoside triphosphates is due solely to RNA polymerase. Although it is reasonable to propose that the nucleases stimulate polymerase by virtue of their ability to hydrolyze the RNA product one may also assume that the nucleases could act by some other

mechanism. It is possible that the nucleases might be protecting polymerase against denaturation or by in some manner affecting the template DNA since it has been shown by Felsenfeld, et al. (16), that pancreatic RNase destabilizes DNA. A good system for studying these possibilities is the polymerase catalyzed synthesis of poly A when ATP is the only nucleotide present (17). Poly A is not affected by either pancreatic RNase or T1 RNase, in addition poly A synthesis is more efficiently directed by denatured DNA than by the native DNA used in these reactions (4,18). Therefore, if the nucleases were in some manner destabilizing the DNA or stabilizing polymerase, we should note a stimulation of $^{32}PP_i$ release. The data presented in Figure 1 show that when one measures $^{32}PP_i$ release under conditions where poly A is synthesized there is no effect of added nucleases since the curves for $^{32}PP_i$ release in the presence or absence of the nucleases are virtually superimposable. Therefore, the mechanism by which the RNases stimulate polymerase activity can be ascribed to their ability to attack sensitive linkages in the RNA synthesized and render it innocuous.

The data presented in Figure 2 demonstrate the effect of nucleases on the time course of RNA synthesis (^{14}C-AMP incorporated into acid-insoluble form) and $^{32}PP_i$ release. In the standard reactions (minus nuclease) one obtains the usual time course of activity. There is an initial rapid phase of RNA synthesis followed by slowing, and finally the reaction stops. The release of $^{32}PP_i$ from UMP-P-^{32}P shows a similar time course, although after one hour of incubation there is an apparent excess of $^{32}PP_i$ released; this may reflect oligonucleotide synthesis by the "clogged" enzyme. When the reaction mixture is incubated in the presence of pancreatic RNase and T1 RNase virtually no RNA accumulates, 0.5 mμ moles of ^{14}C-AMP in acid insoluble material as compared with 7 mμ moles in the control lacking nuclease. However, the release of $^{32}PP_i$ from

UMP-P-^{32}P is markedly stimulated and the kinetics approach linearity in response to the removal of the inhibiting product RNA. The deviation from linearity may be ascribed to end-product inhibition by the accumulation of pyrophosphate which at 2 hours incubation approaches 1 mM.

Previous studies with RNA polymerase from A. vinelandii (1) and other bacteria (4, 19), have shown that spermidine and other polyamines stimulate the native DNA-directed synthesis of RNA. It had been proposed that the polyamines stimulated RNA polymerase by affecting the RNA product so as to render it a less efficient inhibitor (1). If this were a valid supposition then one should expect the polyamines to behave in a manner analogous to nuclease. Figure 3 shows the results of an experiment similar to that in Figure 2 the only difference being the addition of spermidine to the reactions. As expected the polyamine stimulates both ^{14}C-AMP incorporation into RNA and $^{32}PP_i$ release from γ^{32}P-UTP. Although the added pancreatic and T1 RNase degrades RNA formed during the reaction run with spermidine one does not obtain as pronounced a nuclease stimulation of $^{32}PP_i$ release in the reactions run in the presence of spermidine as was noted in those reactions lacking polyamines (Figure 2). The almost equivalent release of $^{32}PP_i$ found in the reactions containing the ribonucleases in the presence or absence of spermidine, probably represents the maximal rate of RNA polymerase activity. Therefore, the polyamines do not act principally by directly affecting RNA polymerase or the DNA template, but the stimulation is elicited indirectly by affecting the RNA product.

Figure 4 shows the results of an experiment in which pancreatic RNase and T1 RNase were added to the reaction at a time when polymerase activity had come to a halt. To demonstrate that RNA polymerase is not appreciably affected by incubation at 37° the reaction is begun in the absence of the template. Upon addition of DNA, RNA synthesis is initiated and follows the characteristic time course. When RNA synthesis stopped γ^{32}P-UTP is added and the mixture divided into two portions, the first (control) receives no further

additions, to the second portion are added T1 and pancreatic RNases and both are incubated at 37°. The control system shows no further RNA synthesis although there is some $^{32}PP_i$ release. This may again represent oligonucleotide synthesis or pyrophosphate exchange with the unlabeled PP_i resulting from the prior synthesis of RNA. The reaction to which the nucleases are added shows only slight residual ^{14}C-labeled RNA and concomitant with the degradation of the RNA there is a marked stimulation of utilization of the added UMP-P-^{32}P as shown by $^{32}PP_i$ release. The results demonstrate that although RNA polymerase is inhibited by the RNA produced during the course of the reaction, this inhibition is not irreversible since the fact that the enzyme is still active can be demonstrated by addition of ribonuclease.

DISCUSSION

The results presented in this paper are consonant with the assumption that the RNA formed during the course of the reaction inhibits RNA polymerase. This proposal initially arose from a consideration of the peculiar kinetics of the DNA-directed synthesis of RNA by RNA polymerase from E. coli (2,3), M. lysodeikticus (4), and A. vinelandii (1). Support for such a mechanism was given by the finding that RNA polymerase could use polyribonucleotides to direct the synthesis of complementary polymers (3,6,20). This indicated that the template site in the enzyme could be occupied by RNA as well as DNA. It was also shown that the native DNA-directed synthesis of RNA by the A. vinelandii polymerase could be inhibited by a variety of polyribonucleotides and that this inhibition could be reversed by spermidine and putrescine (6). These findings were confirmed and extended by Fox, et al. (8), working with the RNA polymerase of M. lysodeikticus. It had been shown previously that S-RNA is an effective

inhibitor of RNA polymerase of E. coli and the studies using the M. lysodeikticus polymerase showed that this inhibition was elicited only if the enzyme was allowed to interact with the S-RNA prior to addition of the DNA. This was consonant with the proposal of Berg, et al. (21), that the binding of RNA polymerase to native DNA is almost irreversible. Fox, et al. (8), showed that mixing S-RNA with polymerase results in a complex of the two components which sediments slightly ahead of free polymerase in a glycerol gradient. It was also possible to demonstrate a complex between MS 2 RNA and polymerase, furthermore this complex was dissociated by the addition of spermidine to the gradient solution. The authors were impressed by the evident irreversibility of the DNA-enzyme interaction and concluded that it was unlikely that the RNA synthesized in the DNA directed reaction could be responsible for the deviation from linear kinetics by competing for the template site on the enzyme.

In contrast to the preceding conclusion, the data presented in the present work would seem to support the contention that RNA product inhibition is the primary cause for the divergence from linear kinetics. By using appropriately labeled substrates it was possible to assay polymerase activity by release of $^{32}PP_i$ under conditions where accumulation of RNA product was prevented. The RNA synthesized in the presence of pancreatic RNase and T1 RNase was degraded to oligonucleotide fragments which had at best a limited ability to inhibit polymerase, so that the release of $^{32}PP_i$ approached linearity. These findings indicate that the RNA synthesized during the course of the DNA directed reaction inhibits RNA polymerase but provide no details as to the means by which this occurs.

The work of Bremer and Konrad (22) with the E. coli RNA polymerase has shown the formation of a complex of template DNA-RNA polymerase and RNA product

which does not dissociate during the course of RNA synthesis *in vitro*. It is possible to demonstrate such a complex with the *Azotobacter* enzyme using retention of the complex by filtration of reaction mixtures at neutral pH on membrane filters (Millipore HA). The RNA formed in the presence of spermidine in the T2-DNA directed reaction is similarly retained on filters with the DNA template so that it is evident that although the organic cation stimulates RNA synthesis it does not do so by dissociating the RNA from the complex.[3] The RNA synthesized by polymerase can be bound at least two sites: The first is the area on the enzyme where it is synthesized, the second site may be that occupied by the DNA template. As the RNA chain lengthens it may, due to its being held in proximity to the template site, bind to it and interfere with the transcription process. The addition of polyamines would not affect binding of the product to the synthetic site and would therefore not dissociate the DNA-enzyme-RNA complex. However, the polyamines may affect the physical conformation of the RNA is some manner as to render it unable to bind to the template site and in this fashion stimulate RNA synthesis. When the reaction is carried out in the presence of ribonucleases, that portion of the RNA bound to the synthetic site at any time during the course of the reaction may not be available to nuclease attack, whereas the 5' end of the elongating chain would be degraded as it left the protected environment of the enzyme. Thus the part of the RNA which would interfere with polymerase activity would be removed as it is formed.

SUMMARY

RNA polymerase directed by a native DNA template shows an initially rapid rate of RNA synthesis which subsequently slows and finally a plateau is established. It has been suggested that these kinetics result from the inhibition of RNA polymerase by the RNA formed during the reaction. The kinetics of the polymerase reaction under conditions where product RNA does not accumulate have been studied. By determining the release of $^{32}PP_i$ from $\gamma^{32}P$-labeled ribonucleoside triphosphates it is possible to assay RNA polymerase in the presence of pancreatic RNase and T1 RNase. When the RNases are added to DNA directed RNA polymerase reactions there is a stimulation of $^{32}PP_i$ release and the kinetics approach linearity. The addition of the nucleases to plateaued RNA polymerase reactions markedly stimulates RNA polymerase dependent $^{32}PP_i$ formation.

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FOOTNOTES

1/ The ^{32}P-labeled nucleotides were synthesized by Mr. William J. Horsley.

2/ The abbreviations used are: TCA, trichloroacetic acid; sRNA, soluble ribonucleic acid.

3/ Unpublished observations.

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Fig. 1

The effect of ribonucleases on the DNA directed synthesis of Poly A by RNA polymerase. Each point represents a standard reaction containing 0.4 units of RNA polymerase and 100 mμmoles of AMP-P-^{32}P (1.2 x 10^5 C.P.M. per μmole) as the only ribonucleoside triphosphate. Where noted 10 μg of pancreatic RNase and 20 units of T1 RNase were added. The reaction mixtures were incubated for the times indicated and were then assayed for release of $^{32}PP_i$

Fig. 2

The effect of ribonucleases on the time course of the RNA polymerase reaction. The standard assay was scaled up 8 fold (to 2 ml.) and contained 6 units of RNA polymerase. In order to determine $^{32}PP_i$ released and RNA synthesis 1 μmole of UMP-P-^{32}P (5 x 10^5 C.P.M. per μmole) and 1 μmole of ^{14}C-ATP (2.5 x 10^5 C.P.M. per μmole) were added in addition to 1 μmole each of CTP and GTP. To the reaction containing the nucleases 80 μg of pancreatic RNase and 160 units of T1 RNase were added. Each point represents 0.1 ml of the reaction removed at the time indicated and assayed for either ^{14}C-AMP incorporated into RNA or $^{32}PP_i$ released.

Fig. 3

The effect of ribonucleases on the time course of the polyamine-stimulated RNA polymerase reaction. The conditions are identical to those given in Fig. 2, with the exception that 0.012 M spermidine was present in the reactions.

Fig. 4

Release of RNA product inhibited polymerase by ribonucleases. The standard assay was scaled up 8 fold (2 ml.) and DNA was omitted. The reaction contained 8 units of RNA polymerase and ^{14}C-ATP (2.5 x 10^5 C.P.M. per μmole), UTP, CTP, and GTP. After 1 hour of incubation 500 μg of calf thymus DNA was added. After an additional 2 hrs. UMP-P-^{32}P (2 x 10^8 C.P.M. per μmole) was added so that the specific radioactivity of the UTP in the reaction was about

2×10^5 C.P.M. per μmole. The reaction was divided into two 0.7 ml aliquots, to one was added 0.1 ml of H_2O, to the other was added 25 μg of pancreatic RNase and 50 units of T1 RNase in 0.1 ml. Each point on the curves represents 0.1 ml of the reaction assayed for ^{14}C-AMP incorporated into RNA or $^{32}PP_i$ released.

Table 1. REQUIREMENTS FOR $^{32}PP_i$ RELEASE FROM γ^{32}P-GTP

Conditions of standard assay with 0.4 units of RNA polymerase. The labeled substrate was GMP-P-^{32}P (2×10^5 C.P.M. per μmole). Where noted 10 μg of pancreatic RNase and 20 units of T1 RNase were added. The reactions were incubated for 120 min. and then assayed for $^{32}PP_i$ released.

COMPONENTS	$^{32}PP_i$ RELEASED (mμmoles)
Complete system	11.5
No Mg^{++}	0.2
No DNA	0.2
No RNA polymerase	0.2
No ATP, CTP, UTP	1.4
Complete + pancreatic RNase + T1 RNase	23.2
No RNA polymerase + pancreatic RNase + T1 RNase	0.2

Table 2. INHIBITION OF $^{32}PP_i$ RELEASE BY ACTINOMYCIN D

Conditions of the standard assay with 0.4 units of RNA polymerase. In those reactions containing γ^{32}P-GTP (1.8×10^5 C.P.M. per μmole) UTP, CTP and ATP were present, where γ^{32}P-UTP (5.4×10^5 C.P.M. per μmole) was the labeled substrate, CTP, GTP and ATP were present. When added the amounts of nucleases used are the same as in Table 1. The reactions were incubated for 120 min. and then assayed for released $^{32}PP_i$.

COMPONENTS	$^{32}PP_i$ RELEASED FROM	
	UMP-P-^{32}P (mμmoles)	GMP-P-^{32}P (mμmoles)
Complete system	14.7	12.7
+ T1 and pancreatic RNases	30.5	23.0
+ actinomycin D (16 μg)	1.0	1.0
+ T1 and pancreatic RNases and actinomycin D (16 μg)	1.0	0.5

Table 3. EFFECT OF RIBONUCLEASES ON $^{32}PP_i$ RELEASE

Conditions of standard assay with 0.3 units of RNA polymerase. The labeled substrate was UMP-P-^{32}P (6×10^5 C.P.M. per μmole). Where noted 10 μg of pancreatic RNase and 20 units of T1 RNase were added. The reactions were incubated for 120 min. and assayed for released $^{32}PP_i$.

ADDITIONS	$^{32}PP_i$ RELEASED (mμmoles)
None	8.8
Pancreatic RNase	15.0
T1 RNase	15.0
Pancreatic RNase + T1 RNase	19.1

Fig 1

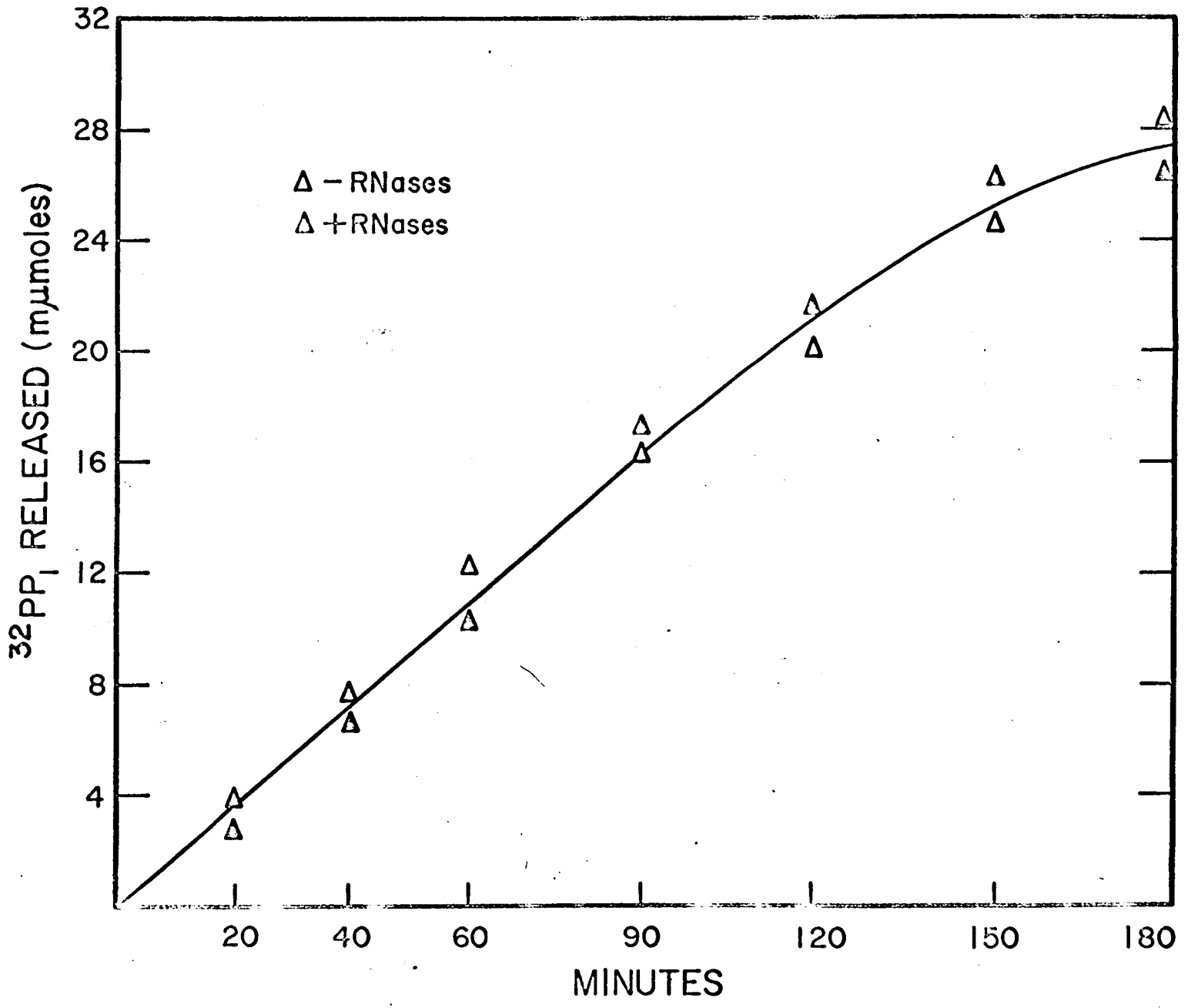

32
28
24
20
16
12
8
4
Δ – RNases
Δ +RNases
$^{32}PP_I$ RELEASED (mμmoles)
20
40
60
90
120
150
180
MINUTES

Fig 2

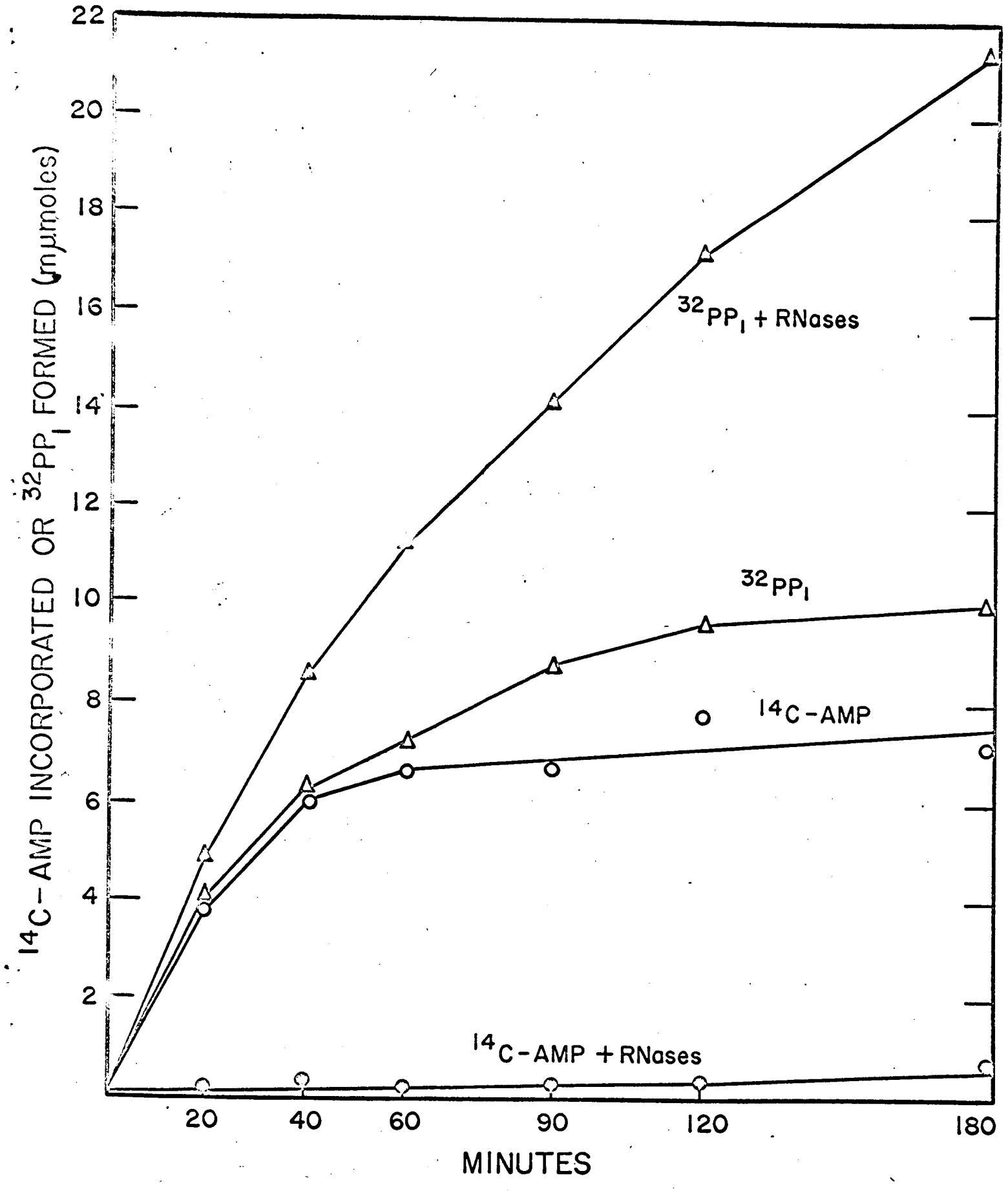

22
20
18
16
14
12
10
8
6
4
2
^{14}C-AMP INCORPORATED OR ^{32}PP$_i$ FORMED (mμmoles)
^{32}PP$_i$ + RNases
^{32}PP$_i$
^{14}C-AMP
^{14}C-AMP + RNases
20
40
60
90
120
180
MINUTES

Fig 3

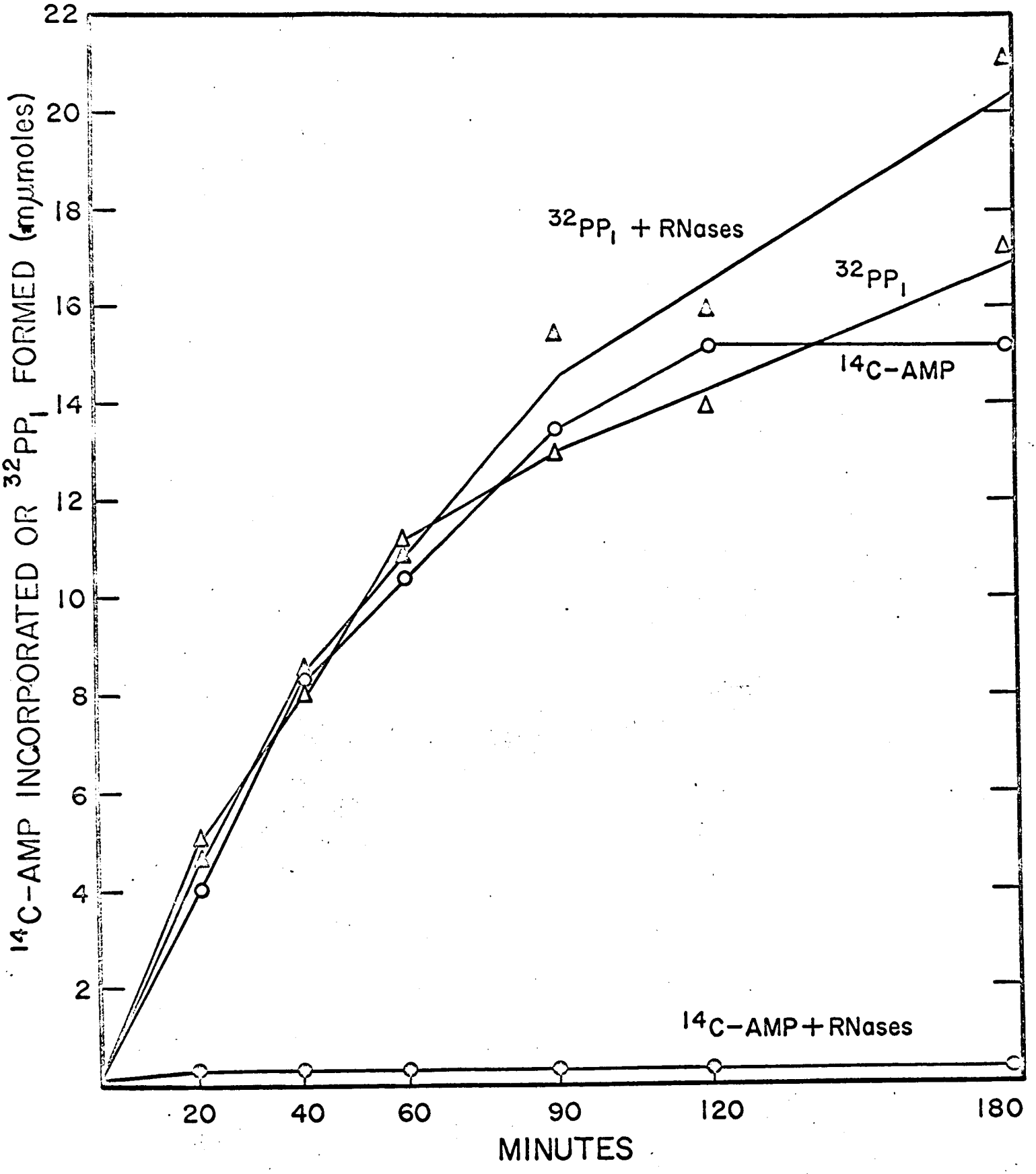
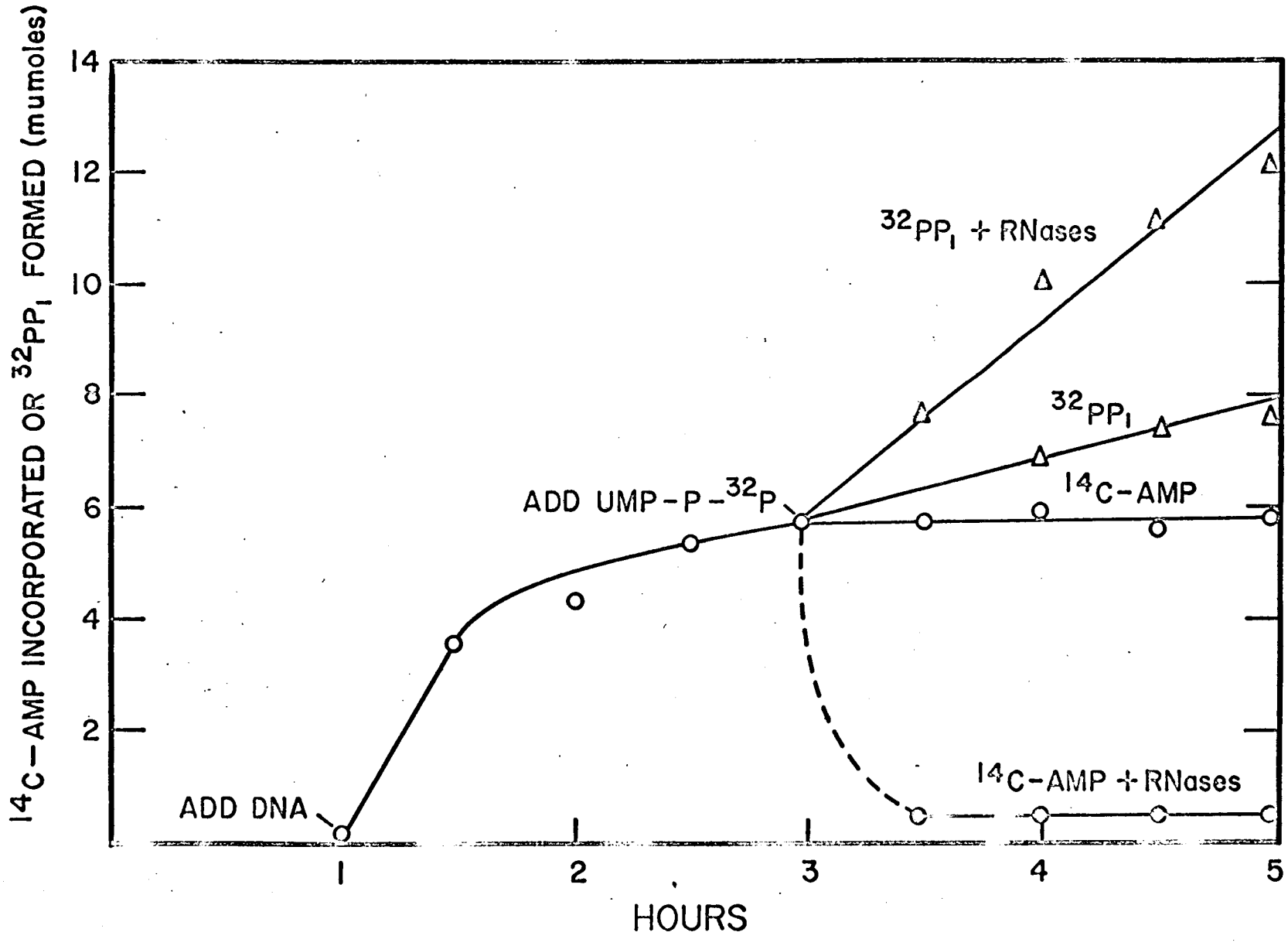

22
20
18
16
14
12
10
8
6
4
2
^{14}C-AMP INCORPORATED OR ^{32}PP$_i$ FORMED (mμmoles)
^{32}PP$_i$ + RNases
^{32}PP$_i$
^{14}C-AMP
^{14}C-AMP + RNases
20
40
60
90
120
180
MINUTES

Fig 4


^{14}C-AMP INCORPORATED OR ^{32}PP$_i$ FORMED (mumoles)
14
12
10
8
6
4
2
1
2
3
4
5
HOURS
^{32}PP$_i$ + RNases
^{32}PP$_i$
^{14}C-AMP
ADD UMP-P-^{32}P
ADD DNA
^{14}C-AMP + RNases