# INHIBITION OF REVERSE TRANSCRIPTASE BY HIGH CONCENTRATIONS OF TRITIUM-LABELLED SUBSTRATES

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Received 3 May 1972

## 1. Introduction

In recent months, various reports [1–3] have indicated that the RNA-dependent DNA polymerase (reverse transcriptase) isolated from avian myeloblastosis virus may be used to obtain DNA copies of 9 S RNA from reticulocytes. For certain experiments it is desirable to obtain a DNA copy of 9 S RNA with the highest specific activity possible. This report describes evidence indicating that reverse transcriptase is inhibited by incubation with deoxyribonucleoside triphosphates in which the total amount of radioactivity is very high, and that the DNA copy obtained under these conditions is much shorter than that obtained with lower concentrations of radioisotope. It is further shown that this problem can be overcome by the addition of certain proteins to the incubation mixture.

## 2. Materials and methods

9 S RNA, which was a gift from Dr. Williamson of this Institute, was obtained from EDTA-treated mouse reticulocyte polysomes and tested for the ability to direct globin synthesis in either the duck lysate or oocyte systems as described in previous reports from this laboratory [4, 5]. Plasma containing avian myeloblastosis virus was the very generous gift of Dr. J.W. Beard, Duke University, USA. The virus was purified, and reverse transcriptase isolated by chromatography on DEAE-cellulose and CM-Sephadex as described by Kacian et al. [6]. Reverse transcriptase obtained from 200 mg virus (wet wt.) was then stored at −20° in 50 ml 50% glycerol, 0.15 M potassium phosphate (pH 8.0) and 1 mM dithiothreitol. This solution had an activity of about 0.3 units/ml [3]. This stock solution was diluted 5-fold with the appropriate incubation mixture for use. Poly rA. poly dT and poly dT (with quoted $S_{w,20}$ values of 8.3 and 2.1 respectively when measured in 0.05 M phosphate, pH 7.0) were obtained from Miles-Seravac; unlabelled deoxyribonucleoside triphosphates from the Boehringer Corporation; and catalase (type C40), peroxidase (type VI) and bovine serum albumin (fraction V) from the Sigma Chemical Company. Tritium-labelled deoxyribonucleoside triphosphates (supplied in 50% ethanol containing 1% ammonium bicarbonate) were obtained from the Radiochemical Centre, Amersham, stored at −20°, and used within 6 weeks of purchase. Bicarbonate was removed from these radioactive solutions, after adding the requisite unlabelled deoxyribonucleoside triphosphates, by mixing them with an excess of Dowex resin (hydrogen-form, AG 50W X 8, which had been washed extensively with 50% ethanol) at 0°, followed by removal of the resin by centrifugation. This procedure was recommended by Dr. Turner of the Radiochemical Centre. The resultant supernatant solution was evaporated to dryness under vacuum. All these manipulations, and incubation of enzyme with substrates, were performed in glassware coated twice with Repelcote and then rinsed with distilled water.

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Table 1
Inhibition by radioactive substrates of the reverse transcriptase.

| | $^3$H-TTP | | Dithiothreitol concentration (mM) | Other additions | Total radioactivity ($\mu$Ci/ml) | pMole TMP incorporated/ml/hr |
|---|---|---|---|---|---|---|
| | Concentration ($\mu$M) | Specific activity (dpm/pmole) | | | | |
| A | 13 | 1,700 | 10 | – | 10 | 56 |
| | | 17,000 | 10 | – | 100 | 39 |
| B | 100 | 247 | 10 | – | 11 | 192 |
| | | 2,470 | 10 | – | 115 | 162 |
| | | | | 370 $\mu$Ci per ml $^3$H-dCTP + 370 $\mu$Ci per ml $^3$H-dCTP | 855 | 112 |
| | | 24,700 | 10 | – | 1150 | 86 |
| C | 50 | 2,100 | 10 | – | 55 | 157 |
| | | 21,000 | 10 | – | 550 | 42 |
| | | 2,100 | 20 | – | 55 | 137 |
| | | 21,000 | 20 | – | 550 | 48 |
| | | 21,000 | 20 | 100 $\mu$g/ml catalase | 550 | 206 |

All incubations were performed at 38° in sealed, nitrogen-flushed tubes which had been coated with Repelcote. The incubation mixture consisted of 50 mM Tris pH 8.2, 50 mM KCl, 5 mM magnesium acetate and dithiothreitol as indicated, 500 $\mu$M (A) or 1 mM (B, C) each of dATP, dCTP and dGTP, and 4 $\mu$g/ml poly rA. poly dT. One fifth-volume of enzyme solution was also added. In (A), the $^3$H-TTP used was batch 13, 7.7 Ci/mole before dilution; in B and C, batch 14, 11.2 Ci/mole before dilution. After the incubation, EDTA was added to give a final conc. of 2 mM and 20–50 $\mu$g of *E. Coli* DNA were added as carrier. The mixture was then frozen at −20°, until it was chromatographed on a G-50 column equilibrated with 0.1 M NaCl; only material excluded from the Sephadex was collected.

## 3. Results and discussion

Table 1 shows that the ability of reverse transcriptase to catalyse the incorporation of $^3$H-TMP into high molecular weight product using poly rA. poly dT as template is dependent on the concentration of substrate TTP. However, at a given substrate concentration, the yield of product is reduced progressively as the specific activity of the TTP is increased. Further data (unpublished) show that the reduction in yield of product correlates with increasing concentration of radioisotope during incubation, rather than with the increasing specific activity of substrate per se. Moreover, addition to the incubation mixture of $^3$H-dCTP and $^3$H-dGTP (which are not used by the enzyme with poly rA. poly dT as template) also reduces the yield of product (table 1B). Since in the latter experiment the incubation mixture also contained a 14-fold excess of unlabelled dCTP and dGTP, it is unlikely that the inhibition of enzyme activity by the radiactive substrates is due solely to radiation damage caused whilst the radioactive substrates are bound to the active site of the enzyme during transcription. It is also unlikely that the phenomenon is due to toxic material introduced into the radioactive solution during treatment with Dowex resin since the resin had been washed with 50% ethanol prior to use. Furthermore the same effect is also observed when the Dowex resin treatment is omitted; however, in this case, the high concentration of bicarbonate in the incubation mixture is a complicating factor.

Two possible explanations for this phenomenon may be suggested:

i) reverse transcriptase is inhibited by some toxic radiation – decomposition product or other toxic material present in the radioactive solutions;

ii) the DNA product destroys itself rapidly due to self-irradiation. The first possibility is supported by

Table 2
Effect of protein in protecting the reverse transcriptase.

| Addition | Reverse transcriptase | pMole dAMP incorporated/90 min/ml/ μg 9 S RNA |
|---|---|---|
| None | + | 0.74 |
| | – | 0.06 |
| Catalase 100 μg/ml | + | 7.9 |
| | – | 0.05 |
| Catalase 10 μg/ml | + | 0.88 |
| | – | 0.06 |
| Bovine serum albumin 100 μg/ml | + | 11.6 |
| | – | 0.07 |
| Peroxidase 100 μg/ml | + | 4.3 |
| | – | 0.08 |

Incubations were performed at 38° in 50 mM Tris, pH 8.2, 50 mM KCl, 10 mM dithiothreitol, 5 mM magnesium acetate, 5 μg/ml 9 S RNA, 1 μg/ml poly dT, 20 μg/ml actinomycin D, 500 μM each of dCTP, TTP, dGTP and 40 μM $^{3}$H-dATP (9.5 Ci/mmole, batch 6). One fifth of enzyme stock solution was then added.

the finding that addition of catalase to the incubation mixture improves the yield of product considerably (table 1C). However, increasing the concentration of dithiothreitol (which might eliminate any peroxides or free-radicals produced by the radiation) has no effect (table 1C). Further experiments showed that this effect of catalase is also observed when 9 S RNA is used as template in the presence of poly dT as primer, and is concentration-dependent (table 2). Very similar results are obtained whichever labelled deoxyribonucleoside triphosphate is used to measure the formation of product. Moreover, the effect of catalase is not due to binding of radioactive substrate to itself (which would cause low molecular-weight material to be eluted from the G-50 column together with high molecular-weight material): for, when catalase is incubated under the same conditions in the absence of reverse transcriptase, no radioactive high molecular-weight material is observed (table 2). Very similar results are obtained when bovine serum albumin, or (to a lesser extent) peroxidase, replace catalase (table 2). These results suggest that the presumptive toxic product is inactivated by a variety of proteins.

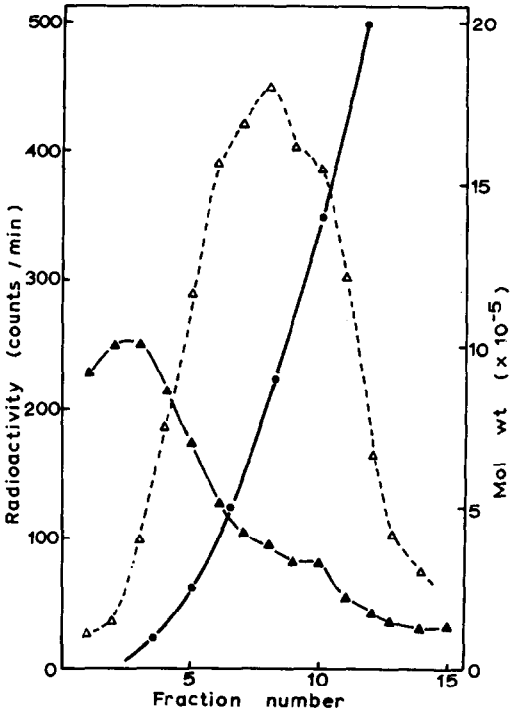


Fig. 1. Effect of catalase on size of product. Incubations were performed as in table 2 except that the concentrations of deoxyribonucleoside triphosphates were as follows: dATP and αdGTP, 500 μM; $^{3}$H-dCTP, 40 μM (9.5 Ci/mmole); $^{3}$H-TTP, 40 μM (11.2 Ci/mmole). In (▲—▲—▲), no extra protein was added; in (△—△—△), 100 μg/ml catalase. After obtaining the high molecular weight fraction from the G-50 eluate, a 1 ml aliquot was adjusted to contain 0.9 M NaCl, 0.1 M NaOH, 2.5% (w/w) sucrose and layered on a linear 20 ml 5–10% (w/w) sucrose gradient containing 0.9 M NaCl and 0.1 M NaOH. The gradient was then centrifuged at 29,000 rpm in the MSE 3 × 25 ml swing-out rotor for 24 hr at 20°. 1 ml fractions were unloaded by upward displacement of the gradient, the refractive indices of fractions measured, and then aliquots counted in Triton X-100 toluene-based scintillator in the presence of 1/20 volume 3 N $HClO_4$. Sedimentation coefficients were calculated as McEwen [7], and the molecular weights (●—●—●) from the data of Studier [8].

Further unpublished experiments have shown that incubation of reverse transcriptase with very high radioactive concentrations of substrate not only decreases the yield, but also the size of product when analysed by sedimentation in alkaline sucrose. Significantly, addition of catalase to the incubation mixtures containing the highest concentrations of radioactivity (of the order of 1 mCi/ml) increases the

size of product dramatically (fig. 1). These experiments prove that the product is in fact high molecular-weight DNA, and disprove the possibility that, at very high specific activity, the DNA product is rapidly destroyed by self-irradiation.

It therefore seems most probable that reverse transcriptase itself is inhibited by the radioactive solutions used in these experiments. Several possible reasons for this inhibition may be considered: i) a chemical inhibitor present in the radioactive solutions as supplied by the Radiochemical Centre; ii) a chemical inhibitor introduced during removal of ethanol and bicarbonate from the radioactive solutions; iii) inhibition by direct radiation damage during transcription; iv) formation of a radiation-induced inhibitor. The first possibility is unlikely since each individual radioactive deoxyribonucleoside triphosphate solution was found to inhibit reverse transcriptase, despite the different methods of preparation in each case. The second and third possibilities are also unlikely, for reasons discussed previously. It is therefore most probable that the radioactive solutions contain some non-volatile radiation-induced inhibitor. Since the inhibitory effect of these radioactive solutions is not abolished selectively by traces of catalase or peroxidase, it is unlikely that the inhibitory material is simply a peroxide.

It is interesting that this phenomenon has not been reported by other workers [1–3]. However, this is readily explained on the basis of the present results. In most other work (e.g. [1, 3]), it has been necessary only to use substrates of low specific activity. In the case [2] where substrates at high radioactive concentration were used, the concentration of the reverse transcriptase during incubation was 90 μg/ml. Under these conditions, the enzyme may "protect" itself.

## Acknowledgements

This work has been supported by grants from The Medical Research Council and The Cancer Research Campaign. The authors are grateful to Dr. G. Birnie for much useful discussion and advice.

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