GLYCEROL INHIBITION OF PURIFIED AND CHROMATIN-ASSOCIATED MOUSE LIVER HEPATOMA RNA POLYMERASE II ACTIVITY

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SUMMARY: The stability of mouse liver hepatoma RNA polymerase II is dependent on the type of buffer, pH and, most importantly, the glycerol concentration of the incubation or storage buffer. Glycerol above 2% or 15% shows a linearly increasing inhibition of enzyme activity with increasing glycerol concentration for purified RNA polymerase II and chromatin-associated RNA polymerase II, respectively. At 25% glycerol the activity of purified enzyme on DNA template was inhibited approximately 50% whereas the chromatin-associated activity was inhibited only approximately 30%. RNA polymerase I activity was not inhibited by glycerol at the concentrations examined.

Since the initial description (1, 2) of the separation of the various eukaryotic DNA-dependent RNA polymerases (RNA nucleotidyltransferase; ribonucleoside triphosphate: RNA nucleotidyltransferase, EC 2.7.7.6) extensive purification and characterization (e.g., 3, 4, 5, 6, 7, 8, 9, 10, 11) have been carried out. In most of the reported studies glycerol is routinely used as a stabilizing agent in the storage and assay buffers. Additionally, in our continuing (12, 13, 14, 15) fractionation and characterization of mouse liver hepatoma chromatin steep glycerol concentration gradients (16, Paul and Duerksen, manuscripts in preparation) are used. During our purification, characterization and use of the mouse liver hepatoma RNA polymerases (Smith and Duerksen, manuscript in preparation), we discovered that glycerol severly inhibited the activity of the RNA polymerase II species whether in purified state on native or single-stranded DNA template or associated with chromatin preparations as endogenous activity. This report describes this inhibition of RNA polymerase II activity by glycerol and indicates the important implications of such an inhibition.

MATERIALS AND METHODS

The source of cells for enzyme preparation and for nuclei for chromatin preparation was from mouse TLT (Taper Liver Tumor) hepatoma (17) grown

in the ascites form in Swiss Webster mice. RNA polymerase activity was obtained from lysed cells as an ammonium sulfate precipitate. This activity was separated into RNA polymerase I and II_A/II_B by DEAE-cellulose chromatography and each of these forms was further purified (specific activities in the range 120-200 units/mg protein) by phosphocellulose chromatography (18, 5, 6) and were kept at -80° in storage buffer containing 50% glycerol. One unit of enzyme is defined as the incorporation of one picomole of UMP per minute at 37° under the conditions described below.

DNA was prepared from nuclei of TLT hepatoma cells by the method of Marmur (19) as modified by Church and McCarthy (20) or by a modification of the high-molecular-weight method of Gross-Bellard et al. (21). Denatured DNA and sheared (15 seconds) DNA were prepared as previously detailed (13, 14).

After TLT hepatoma nuclear preparation (12, 13, 14, 15) by using 0.1% Triton in the cell-homogenizing sucrose buffer and eliminating the 1.7 M sucrose centrifugation step, the chromatin was released from the nuclei by suspension in 10 mM glycine buffer (22, 13, 14, 15). Chromatin was also sheared for 15 seconds (13, 14).

RNA synthesis with the purified enzymes on DNA template was carried out in at least duplicates in 250 µl of a standard incubation medium (1, 2, 18): 80 mM Tris-HCl (pH 7.9), 0.1 mM dithiothreitol (Calbiochem. Calif.), 0.1 mM disodium ethylene diamine tetraacetate, 3 mM MnCl, 50 mM ammonium sulfate and the three nonlabelled nucleoside triphosphates (Calbiochem., Calif.) are at 150 µM while [^{3}H]UTP (Amersham/Searle, Ontario) is at 75 µM and 66 µCi/µmole. When the endogenous RNA polymerase activity of chromatin was measured, a 2.5 ml reaction volume containing sheared chromatin had glycine (pH 7.8) at 40 mM replacing Tris-HCl, MnCl at 1 mM, ammonium sulfate at 288 mM (Smith and Duerksen, manuscript in preparation) and the bases were at 1/5 standard concentration. Glycerol concentrations were as indicated in the figure legends. After stopping the reaction with reaction-stopping mixture at 0° (1, 2), RNA synthesis was determined by measuring the incorporation of labelled nucleotide into acid-insoluble material (13, 14). N-tris(hydroxymethyl)methyl-2-aminoethane-sulfonic acid (TES) was obtained from Calbiochem. (Calif.) while Tris-(hydroxymethyl)aminomethane (enzyme and buffer grade) was obtained from Schwarz/Mann (N.Y.).

RESULTS AND DISCUSSION

The stability of eukaryotic RNA polymerases is dependent on the presence of glycerol in high concentrations in the buffers used (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11). The stability of mouse TLT hepatoma RNA polymerase II incubated at 21° in various buffers at different pHs and at 17 and 50% glycerol concentrations is presented in Figure 1. The monophasic linear decay rate of 35% over a 24 hour period for RNA polymerase II activity in Tris or TES (pH 7.5) containing 50% glycerol, is minimal. When the concentration of glycerol is dropped to 17% the decay rate of enzyme activity increases dramatically and becomes biphasic. Bringing the pH of the incubation buffer much below 7.0 or above 7.5 greatly increases the decay rate of the enzyme activity. The biphasic nature of the decay curve, the inflection

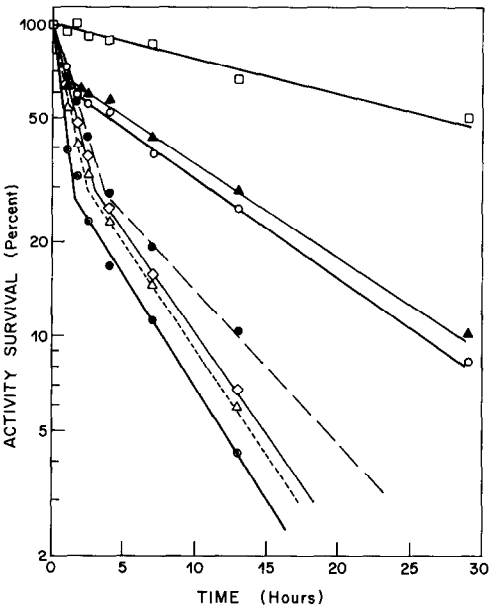
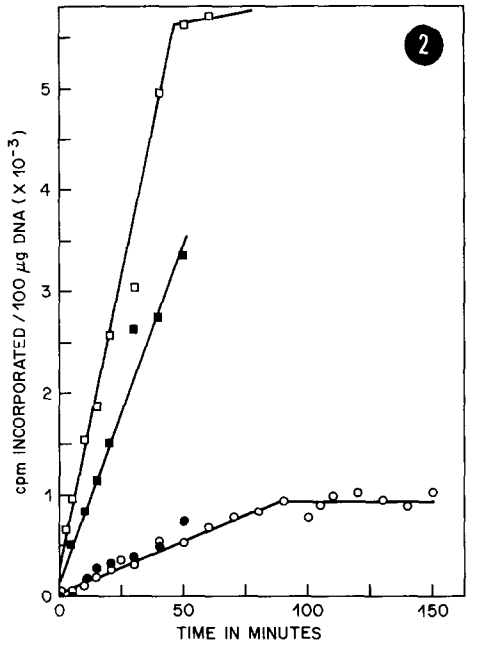


Figure 1. Effect of glycerol, buffer and pH on the stability of mouse TLT hepatoma RNA polymerase II. RNA polymerase II was diluted from the stock enzyme preparation into the indicated buffers at 50 mM at different pHs and containing 0.1 mM EDTA, 1 mM dithiothreitol and glycerol either at 50% or 17% such that 25 µl of preparation contained 7.4 units of enzyme. The mixtures were incubated at 21° and 25 µl removed at the indicated times and assayed for 10 minutes in the standard reaction mix of 0.25 ml containing 25 µg of sheared, denatured TLT hepatoma DNA as template. The incubation preparations for the enzyme contained the following combinations: TRIS or TES pH 7.5 and 50% glycerol (□——□), TES pH 7.0 and 17% glycerol (▲——▲), TES pH 7.5 and 17% glycerol (○——○), TRIS pH 7.9 and 17% glycerol (●—— ——●), TES pH 6.4 and 17% glycerol (◇ —— ◇), TES pH 8.0 and 17% glycerol (△---△), and TES pH 8.5 and 17% glycerol (◎——◎).

point varying with the incubation conditions, is similar to that obtained by Gissinger et al. (9) for the pattern of heat (42°) or urea (1M, 15°) inactivation of calf thymus RNA polymerase II in a buffer containing 30% glycerol. Temptingly these results can be correlated with the known presence of two forms (II_A and II_B) of the enzyme in our partially purified preparation (Smith and Duerksen, unpublished results), their complex subunit make-up and subunit structural differences (9, 10, 11). In any case the stabilizing effect of high concentrations of glycerol on RNA polymerases of mouse TLT hepatoma cells is evident.

Consequently, it is necessary to examine the effect of glycerol at various concentrations on the activity of the several mouse TLT hepatoma RNA polymerases. Figure 2 shows the kinetics of incorporation of UMP by



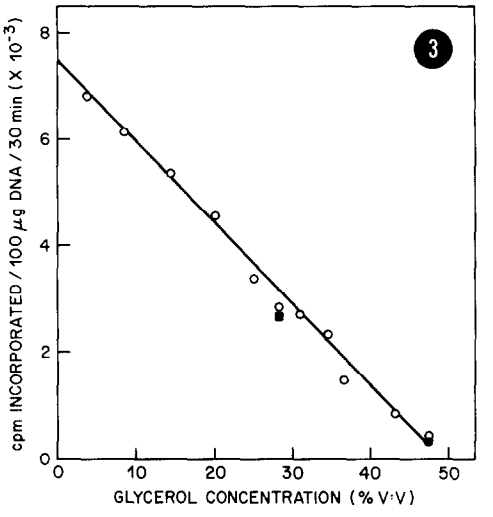


Figure 2. Effect of glycerol concentration on rate of [^{3}H]UTP incorporation by RNA polymerase II. Firstly, in a series of reaction volumes of 0.26 ml 36 μg of sheared, native TLT hepatoma DNA served as template for 2 units of TLT hepatoma RNA polymerase II_A in the presence of 4% glycerol (□——□). At the incubation (37°) times indicated reactions were terminated by the addition of reaction-stopping mixture at 0° and the samples analyzed. Secondly, large reaction mixtures containing, respectively, 28% glycerol (■——■), 48% glycerol (○——○) and 50% glycerol (●——●), had 46 μg of sheared, native TLT hepatoma DNA serving as template for 4.5 units of a mixture of TLT hepatoma RNA polymerases II_A/II_B per a 0.26 ml volume. At the indicated incubation (37°) times 0.2 ml aliquots were removed from each reaction mixture and added to reaction-stopping mixtures at 0° and analyzed.

Figure 3. Effect of glycerol concentration on the activity of RNA polymerase II. Reaction mixtures of a volume of 0.26 ml contained 46 μg sheared, native TLT hepatoma DNA serving as template for 4.5 units of a mixture of TLT hepatoma RNA polymerases II_A/II_B (○——○). Glycerol concentrations as indicated were present in their corresponding reaction mixtures. The reaction was initiated by the addition of enzyme and terminated at 30 minutes incubation (37°) by the addition of reaction-stopping mixture at 0° and the samples analyzed. ■ and ● are the 30 minute values for 28% and 48% glycerol taken from Figure 1.

RNA polymerase II_A in 4% glycerol compared to the rate of incorporation by RNA polymerase II_A/II_B in 28, 48 and 50% glycerol. Glycerol decreases the rate of UMP incorporation and at the higher concentrations incorporation ceases at approximately 85 minutes. When glycerol concentrations are varied between 4 and 50% (Figure 3), it is evident that the inhibition of RNA polymerase II activity is directly proportional to the glycerol concentration; at 25% glycerol the activity is inhibited approximately 50%. As shown by the results presented in Figure 4, RNA polymerase I is unaffected by glycerol concentrations within the range examined. This lack of inhibition of RNA polymerase I activity is also evident on double-stranded DNA as template (Data not shown). These results further show that glycerol in a concentration-dependent fashion inhibits RNA polymerase II whether the

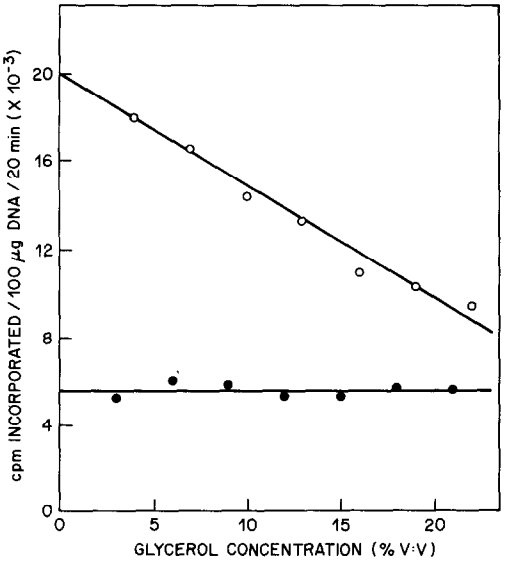


Figure 4. Effect of glycerol concentration on the activity of RNA polymerase I and II on single-stranded, sheared DNA. Reaction mixtures of a volume of 0.26 ml contained 23 µg of sheared, denatured TLT hepatoma DNA serving as template for 2.4 units of TLT hepatoma RNA polymerase I (●—●) or 3.6 units of a mixture of TLT hepatoma RNA polymerases II_A/II_B (○—○). Glycerol concentrations as indicated were present in the corresponding reaction mixtures. The reactions were initiated by the addition of enzyme and terminated at 20 minutes by the addition of reaction-stopping mixture at 0^{o} and the samples analyzed.

template is single-or double-stranded DNA. In addition, this glycerol inhibition of RNA polymerase II activity is demonstrable for the enzyme associated with chromatin obtained from lysed mouse TLT hepatoma nuclei (Figure 4). The cryptic portion of this enzyme activity is expressed only at relatively high ammonium sulfate concentrations (23, 24, Smith and Duerksen, unpublished results) and the majority of the activity is due to α-amanitin-sensitive RNA polymerase II form (Smith and Duerksen, unpublished results). Glycerol up to a concentration of approximately 15% had no inhibitory effect on this enzyme activity. Above this concentration the degree of inhibition is directly proportional to the glycerol concentration such that at 35% glycerol concentration there is approximately 50% inhibition of this RNA polymerase activity.

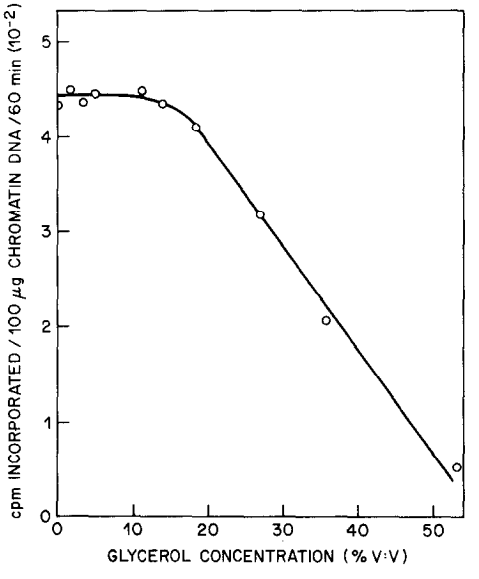


Figure 5. Effect of glycerol concentration on the activity of chromatin endogenous RNA polymerase activity. Reaction mixtures of a volume of 2.5 ml contained the described proportions of ingredients (see materials and methods) but replaced Tris with glycine at 40 mM concentration. Ammonium sulphate at a final concentration of 288 mM for endogenous RNA polymerase activity, was present in each reaction mixture as well as the indicated glycerol concentrations. The reactions were initiated by adding 0.2 ml (200 µg chromatin DNA) of a sheared TLT hepatoma chromatin preparation. After 60 minutes incubation (37°) reaction-stopping mixture at 0° was added and each sample analyzed.

In conclusion, glycerol has a dramatic inhibitory effect on mouse TLT hepatoma RNA polymerase II whether in a purified form or still associated with the chromatin obtained from lysed nuclei. This inhibition is demonstrable whether single-or double-stranded DNA is used as template. The inhibitory effect of glycerol is reversible since diluting out the glycerol restores the enzyme activity (data not shown). At what level of transcription this glycerol inhibition is manifested cannot be determined from these results. It would now be important to determine whether or not glycerol has similar inhibitory effects on RNA polymerases obtained from other eukaryotic cells. If so, the implications to the quantitative evaluation of RNA-polymerase-activity measurements, where varying amounts of glycerol are present, are rather great. The details of the purification and characterization of the RNA polymerases of mouse liver TLT hepatoma cells and their use in estimating template capacities of chromatin fractions, will be published elsewhere.

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