ANALYSIS OF THE EARLY AND LATE PRODUCTS OF THE DNA POLYMERASE OF FRIEND MURINE LEUKEMIA VIRUS WITH ACTINOMYCIN D

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1. Introduction

It is now fairly well accepted that transcription of RNA in RNA tumor viruses proceeds in three steps:

$$\text{RNA} \xrightarrow{\text{I}} \underset{\text{DNA}}{\text{RNA}} \xrightarrow{\text{II}} \underset{\text{DNA}}{\text{DNA}} \xrightarrow{\text{III}} \underset{\text{DNA}}{\text{DNA}}$$

Of these three reactions, actinomycin D is presumed to inhibit only stage II and III leading to the accumulation of single-stranded DNA [1]. Such DNA from actinomycin inhibited reactions has been used in hybridization studies to characterize the extent and specificity of the single-stranded DNA transcribed *in vitro* from RNA preparations containing 9–10S globin mRNA [2,3].

However, the rate reaction profile in the presence or absence of actinomycin D originally obtained for the Rous Sarcoma virus (RSV) endogenous reaction [1] would indicate that all three reactions are inhibited by actinomycin D. This led us to reexamine the reaction with actinomycin D in Friend Murine Leukemia virus [4], an RNA tumor virus maintained in this laboratory in infected mouse tissue culture cells [5]. This virus contains RNA dependent, DNA dependent and hybrid (poly A-oligo dT) dependent activities and displays the enveloped morphology characteristic of type C murine leukemia viruses [6].

The results would indicate that: 1) actinomycin D at 30 μg/ml final concentration inhibits the RNA dependent DNA synthesis in the Friend virus *in vitro* system and 2) the product from actinomycin D inhibited reactions is of lower average density and molecular weight than the normal reaction product. Such results suggest caution in the interpretation of hybridization studies utilizing 'single-stranded' DNA obtained from actinomycin D inhibited reactions.

2. Methods

Experimental details are given in the legends to the figures and tables.

3. Results

The rate reaction profiles (fig. 1) establish that actinomycin D retards the transcription of RNA (stage I) as well as DNA (stage II and III). This is true even if the endogenous reaction is supplemented with RNA purified from Friend Murine Leukemia virus.

The 20–30 min and 4 hr reaction products obtained from RNA supplemented reaction mixtures of actinomycin D inhibited and control experiments were analyzed on cesium sulfate density gradients (fig. 2). Both native and alkali hydrolyzed products were compared. With actinomycin D the appearance of products banding with RNA is delayed (compare fig. 2e and 2b). In addition, comparison of the densities of the native and alkali treated products would indicate that actinomycin D preferentially restricts synthe-

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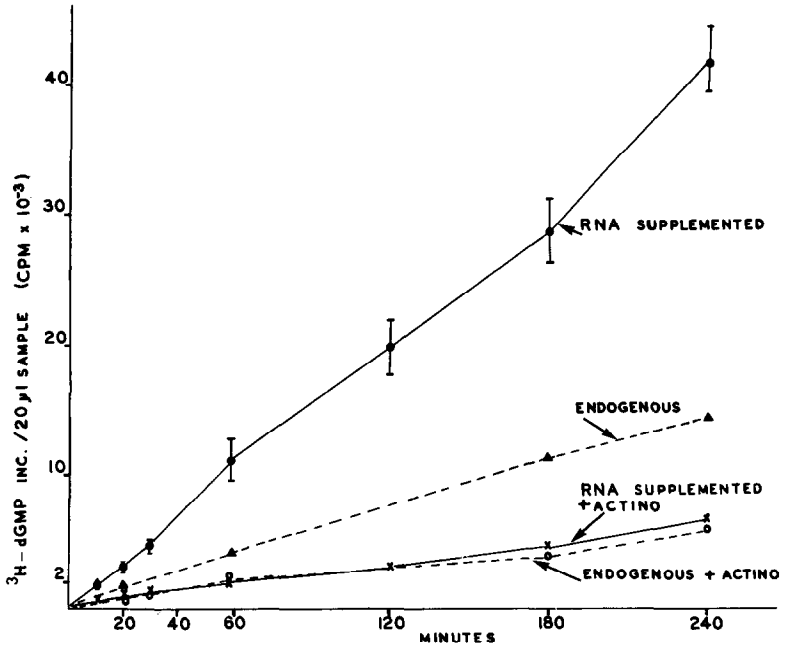
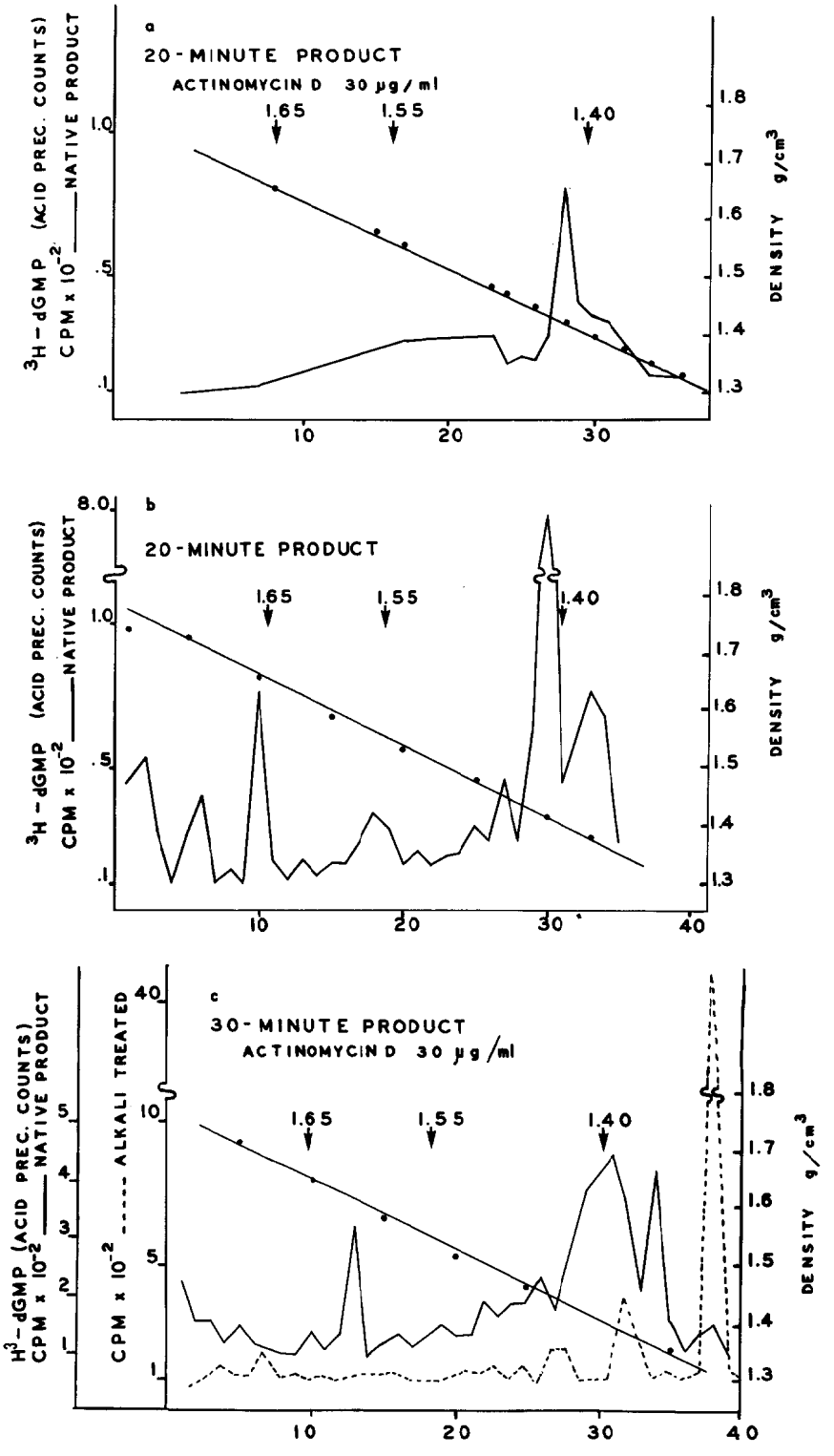
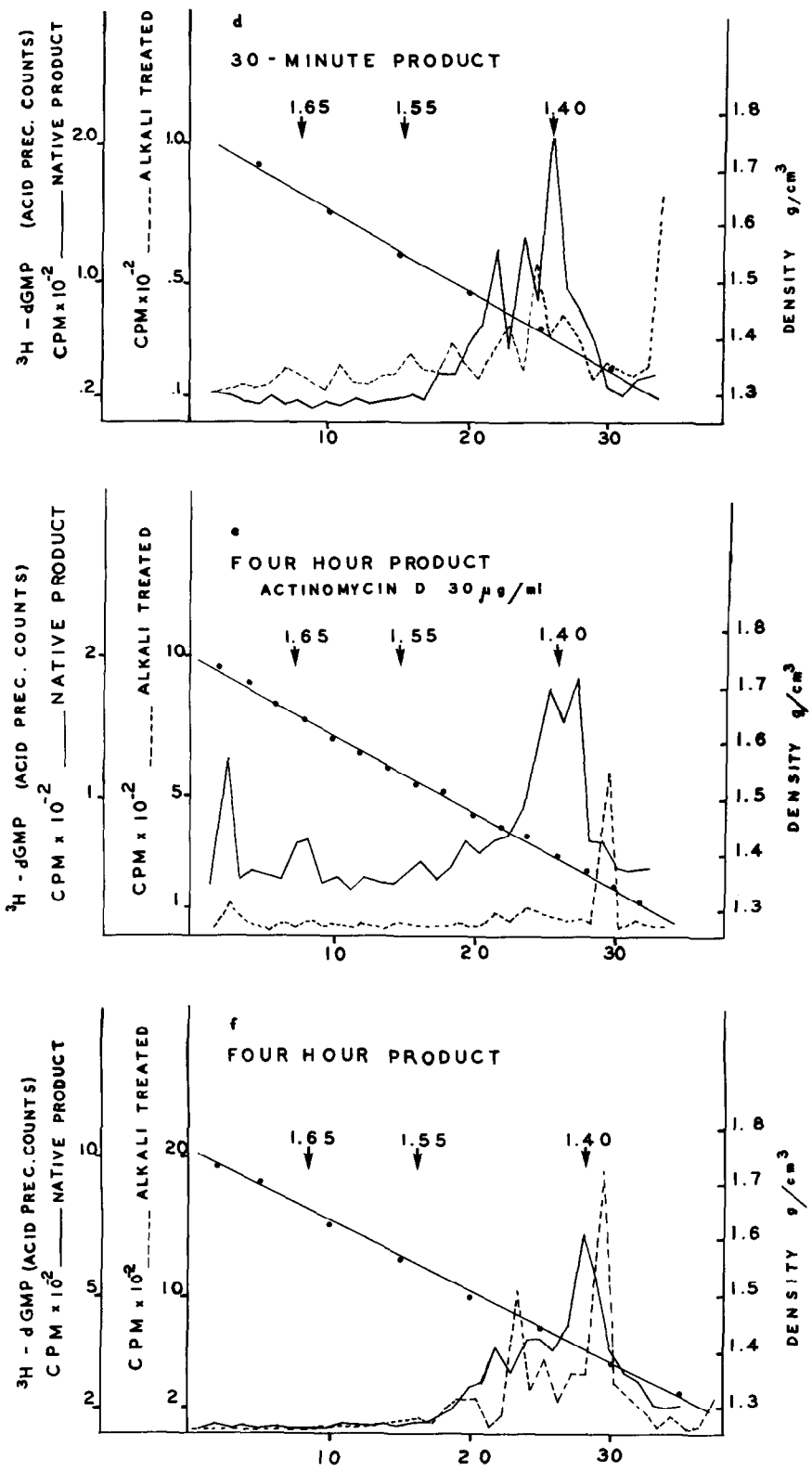


Fig. 1. Effect of actinomycin D on the endogenous and RNA supplemented reaction rates of Friend virus DNA polymerase. Average of three separate experiments with the deviations shown for the RNA supplemented reaction. Six times the standard assay mixture was prepared and incubated at 39°C. At the times indicated 20 µl samples were taken in duplicate and were processed on DE-81 2 × 2 cm^2 paper discs [7]. A standard assay mixture (55 µl) contained 20 mM Tris–HCl, pH 7.8 (37°C); 80 mM NaCl; 2.0 mM Dtt, 1.0 mM manganese acetate, 1.0 µCi [^{3}H]dGTP, 13.4 Ci/mmole, 0.45 mM each dATP, dCTP, dGTP, 0.02% Triton-X100 and 27 µg crude viral enzyme [6], and where indicated 30 µg/ml final concentration of actinomycin D in water and 3.4 µg Friend virus RNA extracted and purified by a modified SDS–phenol method [8, 9] (phenol–10% cresol–1% hydroxyquinoline equilibrated with standard buffer [9]).

sis to products of lighter buoyant density (A-rich), even if a shift of 20–40 mg/cm^3 for actinomycin binding to DNA is taken into account [10]. It should be noted that the radioactive label employed was [^{3}H]dGTP and any product of a given density must contain guanosine in order to be recorded. Therefore, the relative densities and the shifts in density after alkaline hydrolysis are more significant than the amounts of low density products, which must greatly exceed those actually recorded.

There is one apparent discrepancy in the data. Even if actinomycin inhibits all three steps of the reaction, one should find early in the reaction counts banding in the RNA region and the RNA-DNA hybrid region of the gradient. Only products banding in the DNA region of the gradient and some products banding as a broad zone in the RNA–DNA hybrid region of the gradient are found (fig. 2a). However, the product banding in the DNA density region appears to be a RNA–DNA hybrid rich in dA (ρ = 1.379) [11] as evidenced by the shift toward the lower density following alkaline hydrolysis (fig. 2c and 2e). From this it can be inferred that the native product banding in the DNA region contains hybrids rich in rU:dA and possibly others of more complex constitution [11, 12] One conclusion to be drawn from these results might be that because of presumed actinomycin binding to the template, the polymerase preferentially copies the more accessible regions of RNA's that have become separated from the bulk of the 60–70 S RNA through nucleolytic activity. These low molecular RNA's most likely contain sequences that are low in G–C content. A second factor influencing the results obtained concerns one of experimental bias. Products initially resulting from the transcription of low molecular weight RNA's would not be detected if they were removed prior to cesium sulfate density centrifugation [12–15]. In contrast, Riman and Beaudreau [16],


a
20-MINUTE PRODUCT
ACTINOMYCIN D 30 µg/ml
1.65
1.55
1.40
^{3}H-dGMP (ACID PREC. COUNTS)
CPM x 10^{-2} ——NATIVE PRODUCT
DENSITY g/cm^3
b
20-MINUTE PRODUCT
1.65
1.55
1.40
^{3}H-dGMP (ACID PREC. COUNTS)
CPM x 10^{-2} ——NATIVE PRODUCT
DENSITY g/cm^3
c
30-MINUTE PRODUCT
ACTINOMYCIN D 30 µg/ml
1.65
1.55
1.40
H^3-dGMP (ACID PREC. COUNTS)
CPM x 10^{-2} ——NATIVE PRODUCT
CPM x 10^{-2} ----- ALKALI TREATED
DENSITY g/cm^3


d
30 - MINUTE PRODUCT
1.65
1.55
1.40
^{3}H - dGMP (ACID PREC. COUNTS)
CPM x 10^{-2} ——— NATIVE PRODUCT
CPM x 10^{-2} ------ ALKALI TREATED
DENSITY g/cm^3
e
FOUR HOUR PRODUCT
ACTINOMYCIN D 30 µg/ml
f
FOUR HOUR PRODUCT

who studied thymidine labeled material produced by Avian Myeloblastosis virus in tissue culture selected low molecular weight material from the top of glycerol gradients. 4–12 S RNA's are found in RNA tumor viruses and the amounts may vary with the 'age' of the virus [17]. In the present study of the *in vitro* DNA synthesis by Friend virus polymerase no attempt was made to select either low or high molecular weight material.

In control experiments, products banding with RNA, at RNA–DNA hybrid density, and at DNA densities have appeared at 20 min (fig. 2b), but have been converted to largely alkali resistant products at 30 min and 4 hr (fig. 2d and 2f). This indicates that after 30 min the reaction has proceeded well into stage II and III and this is what would be expected during endogenous synthesis [12]. Actinomycin D inhibited reactions, on the other hand, fail to complete these later stages of synthesis at 4 hr (compare fig. 2e and 2f). This would be expected if actinomycin inhibited stage II and III of the reaction sequence. In addition, heteropolymeric regions of RNA do not seem to be transcribed in the presence of actinomycin. Hence, low density products predominate even after 4 hr of synthesis (fig. 2e, alkali treated product). Products from normal and actinomycin inhibited reactions can be shown to rehybridize to viral RNA (not shown).

Finally, products from actinomycin D inhibited reactions are apparently of lower average molecular weight than normal products. This is indicated by the fact that only 34% of the cpm from a 4 hr reaction mixture which absorb on DE-81 paper are TCA precipitable as compared to better than 50% for the control reaction (table 1).

Table 1
Comparison of average size of control and actinomycin D inhibited reaction product of the Friend virus DNA polymerase.

Reaction mixture	Counts/min from 4-hr reaction		
	DE-81 retained	TCA precipitable	% TCA precipitable
Control	4 300	2370	55%
Plus actinomycin D	3 100	1045	34%

Reaction mixtures were prepared as indicated in the legend to fig. 1. After 4 hr comparable amounts (cpm when processed on DE-81 paper) from control and actinomycin D inhibited reaction were tested for TCA precipitability.

4. Discussion

The results reported here suggest that actinomycin D (30 μg/ml) inhibits the RNA directed DNA synthesis of Friend virus *in vitro*. This contention is supported by time–course studies and by the analysis of the reaction products on cesium sulfate gradients. These results are consistent with previous observations that the early synthesis of DNA by RNA tumor viruses (MSV, feLV, AMV [1, 18] is less actinomycin sensitive than the later reaction. The difference is one of interpretation. Our results indicate that only the synthesis of products low in G–C content is insensitive to actinomycin inhibition.

In the presence of actinomycin D the Friend virus DNA polymerase might be prevented from copying heteropolymeric regions of the 60–70 S viral RNA. Because of presumed actinomycin binding to the template, the polymerase preferentially copies the more accessible regions of RNA's that have become separated from the bulk of the 60–70 S RNA. These more accessible regions may contain sequences of rU. This

Fig. 2. Cesium sulfate density gradient analysis of *in vitro* products of Friend virus DNA polymerase. Reaction mixtures were prepared as described in the legend to fig. 1. Samples, generally no less than 50 μl, were taken at the times indicated and were extracted by a micro-phenol–cresol extraction method equilibrated with standard buffer (as in fig. 1). Phase separation was accomplished in 100 μl siliconized capillary tubes. After three extractions at room temperature, one half of each sample was made approximately 0.3 N with NaOH, hydrolyzed for 18 hr at 37°C and then neutralized with HCl (alkali-treated sample). The other half was stored at −20°C until next day (native sample). Both samples were then diluted with 2 mM EDTA to 1.7 ml and an equal volume of saturated Cs_2SO_4 was added and the gradients centrifuged to equilibrium in a SW56 rotor at 30 000 rpm for 64–70 hr [3]. Drop fractions were collected from the bottom of each tube and densities determined on every fifth fraction. TCA precipitable counts were determined after adding 500 μg soluble yeast RNA as carrier and collecting the precipitate on Whatman GF/C glass filters. Precipitates were washed three times with cold 5% TCA–5% sodiumpyrophosphate, twice with 98% ethanol, dried and counted in an SL-30 intertechnique scintillation spectrometer for 10 min in a toluene based fluor.

conclusion is based on the early appearance of a product which on alkaline hydrolysis behaves like a RNA–DNA hybrid rich in dA. Probably no further significance should be attached to the appearance of these early products, although it is of interest that poly-U regions are located at the 3′-termini of the RNA subunits of several RNA tumor viruses and they appear to be the regions where transcription by the polymerase is initiated [19].

Two phases of DNA synthesis in RNA tumor viruses have been observed by other workers. Studies with the Avian Myeloblastosis virus (AMV) have shown that A–T rich DNA products predominate after labeling periods in tissue culture of less than 2 hr, while after longer labeling periods (more than 7 hr) G–C rich DNA products appear in the virus [16]. These authors conclude that there may be two kinds of template present in the viral cores. The results from the *in vitro* transcription of Friend virus RNA reported here are in agreement with these *in vivo* results.

There is some precedent for the preferential copying of certain regions of a template by a polymerase in the presence of actinomycin. For example, a portion of the DNA-directed poly-A and poly-U synthesis by RNA polymerase is actinomycin insensitive [20]. The small amount of G–C present in *Cancer borealis* (crab) DNA renders it only partially susceptible to inhibition by actinomycin, which in this case, affects the rate of synthesis but not the base composition of the product [20]. It might be inferred from these reports that the RNA-directed poly-dA and poly-dT synthesis by the reverse transcriptase of an RNA tumor virus is also actinomycin insensitive and that some guanosine residues can be incorporated provided they occur in sequences that are not favorable to the binding of actinomycin [21]. The data presented in fig. 2 support this. The radioactively labeled [^{3}H]dGTP is incorporated in control and actinomycin inhibited reactions, but high molecular weight products rich in G–C fail to appear in the presence of actinomycin.

The effects of actinomycin D on RNA-directed and DNA-directed synthesis has been studied in Rauscher Murine Leukemia virus (RMLV) [22]. The concentrations of actinomycin required for 50% inhibition of the RNA, denatured DNA and native DNA dependent reactions were 35, 17, and 6 μg/ml respectively, while 100, 50, and 11 μg/ml were required for 90% inhibition. These concentrations are substantially lower than those required for the inhibition of Murine Sarcoma virus (MSV) which required 50 μg/ml for 50% inhibition of the endogenous reaction, while only 75% inhibition could be achieved with concentrations as high as 100–150 μg/ml actinomycin D. It is possible that in Murine Leukemia viruses (Rauscher and the related Friend virus) the RNA directed DNA synthesis is more sensitive to actinomycin inhibition. About 75% inhibition at 30 μg/ml actinomycin D was obtained with the endogenous Friend virus reaction reported here and nearly 90% inhibition when a purified enzyme preparation was used (unpublished observation).

The extent to which actinomycin might be expected to inhibit the RNA dependent step would of course depend on whether tumor virus RNA can bind actinomycin D and this in turn would be a function of the base composition as well as the secondary structure of the RNA. The interaction of actinomycin D with RNA has been observed previously [23–37], although actinomycin does not bind to RNA in the A-type helical configuration (for example tRNA [28]) but can bind to RNA presumably lacking this type of secondary structure. Murine Leukemia virus RNA directly viewed by electron microscope showed long extended linear molecules, which are converted to collapsed structures after DMSO or urea treatment [29]. Such an unusual configuration for isolated RNA in 0.1 M NaCl suggested a structural form with extended hydrogen bonded regions [29]. It would be of interest to investigate the secondary structure of RNA and RNA–DNA hybrids from different tumor viruses and to measure their potential to bind actinomycin more directly.

Because the product from actinomycin D inhibited transcription of Friend virus RNA is of lower average density and apparent molecular weight, it is clear that actinomycin D cannot be used indiscriminately for the preparation of DNA complements from RNA. The extent and nature of the inhibition of the DNA dependent step by actinomycin also deserves reevaluation and further study.

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