MULTIPLE FORMS OF DNA-DEPENDENT RNA POLYMERASE FROM INSECT TISSUE

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1. Introduction

DNA-dependent RNA polymerases have been recently isolated from a variety of eucaryotic tissues such as rat liver [1, 2], rat prostate [3], calf thymus [4], amphibian tissues [5] and from HeLa cells [6], among others. A common feature of all these enzyme preparations is the existence of multiple forms differing in ionic and template requirements, sensitivity towards α-amanitin, subunit structure, molecular size and intranuclear localization (for references see [7]). In this paper we describe the extraction of RNA polymerase from the integument of larvae of the blowfly *Calliphora erythrocephala* and the demonstration of the existence of multiple forms of the enzyme also in this insect tissue.

2. Materials and methods

C. erythrocephala Meigen larvae were reared on bovine meat at 23° and 50% relative humidity. Under these conditions the larval life lasts approx. 8 days.

^{3}H-UTP (1 Ci/mmole) was purchased from The Radiochemical Centre (Amersham, England). ATP, GTP, CTP, UTP, creatinephosphate and creatine phosphokinase (EC 2.7.3.2) were supplied by Boehringer (Mannheim, Germany). DEAE-cellulose was obtained from Whatman (London) and Sephadex from Pharmacia (Uppsala, Sweden). All other chemicals were reagent grade from Merck (Darmstadt, Germany). α-Amanitin was a generous gift of Prof. Th. Wieland (Heidelberg), the rifamycin derivatives A-05 and A-013 were kindly provided by Dr. L. Silvestri (Fa. Lepetit, Milano), Lucanthron by the Wellcome Research Foundation (London) and Lasiocarpin by Dr. Culvenor, CSIRO (Melbourne). DNA from rat liver was prepared as previously described [8], calf thymus DNA was from Calbiochem (Los Angeles) and T4 DNA was kindly provided by Prof. K.H. Seifart.

2.1. *Extraction of RNA polymerases*

The method is in principle based on the work of Kedinger et al. [4] as modified by Gallwitz [9] for the extraction of histone-specific methyltransferases and by Seifart et al. [10] for the extraction of rat liver RNA polymerase. 8 Days old larvae were thoroughly washed with tap water and then with medium A consisting of 0.067 M Tris, 1 M $(NH_4)_2SO_4$, 10 mM $MgCl_2$, 0.25 mM EDTA-Na_2, 1 mM mercaptoethanol, and 10 mM NH_4Cl, 20% glycerol, pH 7.9. The larvae were dried on blotting paper and then pressed between filter papers in order to separate the integument from the rest of the tissues and the hemolymph. The integuments from 30–50 g larvae were then homogenized with an Ultraturrax for 2 min in medium A (150 ml), the homogenate stirred with a magnetic stirrer for 1 hr and then centrifuged at 48,000 *g* in a Sorvall-RC-2-B centrifuge for 20 min. The supernatant was then made 3.5 M in respect to ammonium sulfate, further stirred for either 3 or 12–14 hr and then centrifuged for 20 min at 48,000 *g*. The pellets were taken up in medium B (which is medium A without ammonium sulfate) and submitted to chromatography on Sepha-

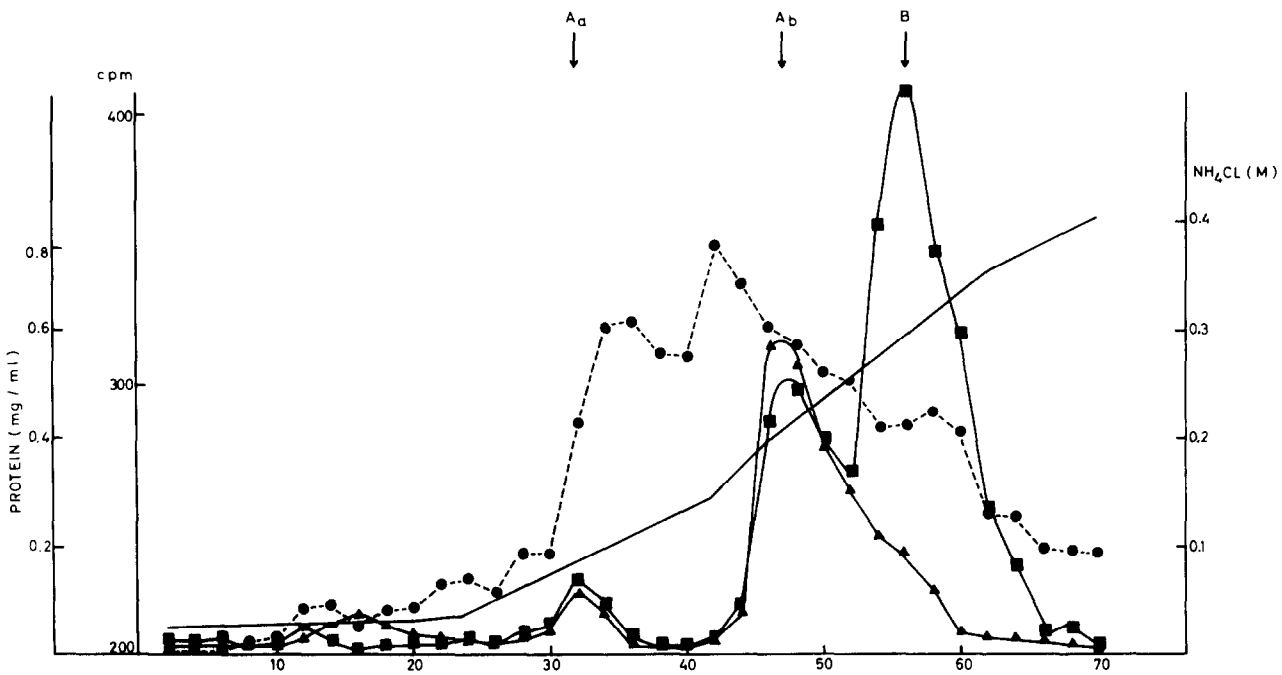

Fig. 1. DEAE-cellulose column chromatography of RNA-polymerases from the integument of 8 days old larvae. For experimental details see Methods. 3 hr exposure to 3.5 M ammonium sulfate. 75 μl aliquots of each fraction have been tested in the assay (150 μl) described in methods. The cpm indicated represent acid-precipitable material in 100 μl aliquots of the incubation mixture. (●- - ●- - ●): proteins; (■—■—■): test without α-amanitin; (▲—▲—▲): test in the presence of α-amanitin (1.3 μg/ml).

Table 1
Transcription by DNA-dependent RNA polymerase forms A_a, A_b and B on different templates.

	A_a (%)	A_b (%)	B (%)
Rat liver DNA			
native	100	100	100
denatured	138	203	325
Calf thymus DNA	21	58	59
T4 DNA	36	33	35

20 μg DNA/150 μl incubation mixture as described in methods. Transcription on native rat liver DNA is taken as 100%.

dex G-25 coarse equilibrated with medium B in order to separate the ammonium sulphate from the proteins.

2.2. *DEAE-cellulose chromatography*

The protein solution was then applied onto a DEAE-cellulose (Whatman DE-32) column equilibrated with medium B. The column was washed with medium B and eluted by a linear gradient of 0.01–0.4 M NH_4Cl (usually 300 ml, fractions 4–5 ml) in medium B.

2.3. *Sucrose gradient centrifugation of RNA polymerase*

A linear 5–20% sucrose gradient in medium B containing 10% glycerol instead of 20% was used. Centrifugation was carried out for 15 hr at 36,000 rpm with an SW 41 rotor in a Beckman L2 or L65 centrifuge. Fractions of 6 drops were collected by piercing the bottom of the tubes with a needle.

2.4. *Standard test for RNA polymerase activity*

The *in vitro* system consisted of 0.2 μmoles each of ATP, GTP and CTP, 0.002 μmoles of UTP, 1.0

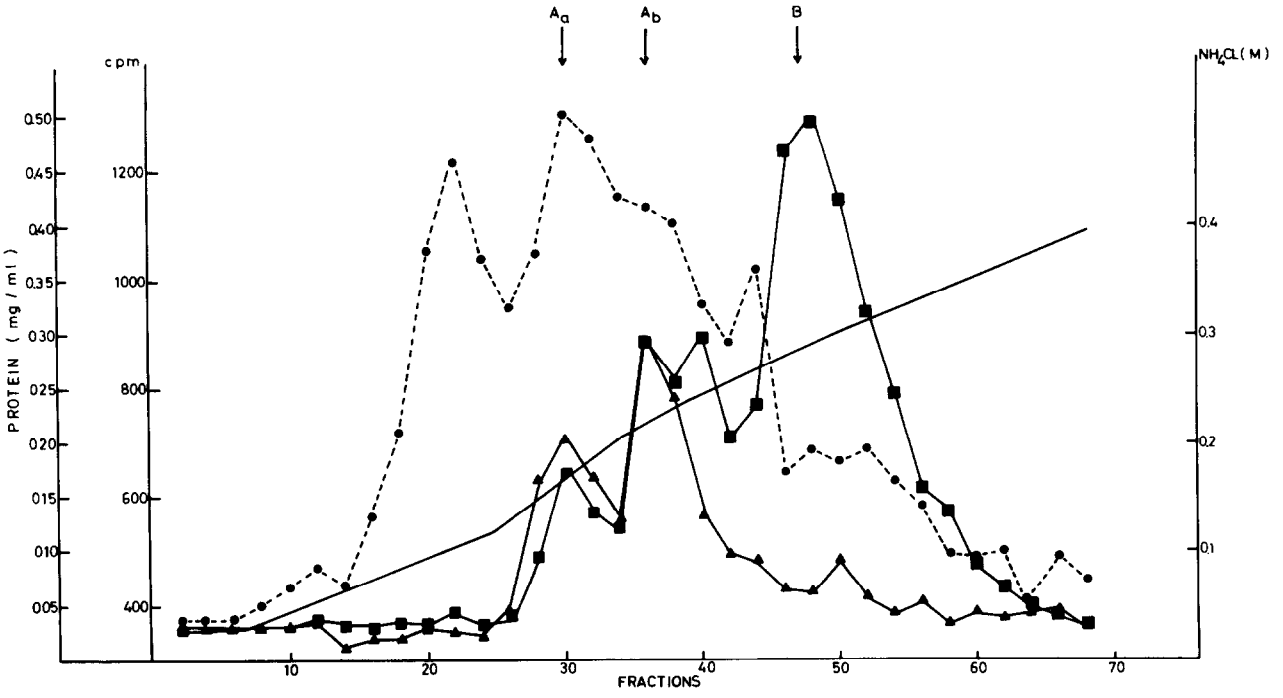

Fig. 2. DEAE-cellulose column chromatography of RNA polymerases under the same experimental conditions as in fig. 1 except a 12 hr exposure to 3.5 M ammonium sulfate. (● - - ● - - ●): proteins; (■—■—■): test without α-amanitin; (▲—▲—▲): test with α-amanitin.

Table 2
Effect of various inhibitors on DNA-dependent RNA polymerase A_a, A_b and B.

	A_a (%)	A_b (%)	B (%)
α-Amanitin	102	104	4 (see fig. 5)
Rifamycin	101	96	101
A-05	76	63	84
A-013	40	63	74
Lucanthron	69	76	80
Lasiocarpin	91	94	92

Inhibitors were present in the incubation mixture in a concentration of 100 μg/ml. The inhibitory effect is expressed as percent of control.

μCi ^{3}H-UTP, 1.5 μmoles creatine phosphate, 5 μg creatinephosphokinase, 1.5 μmoles mercaptoethanol, 10 μmoles Tris-HCl, pH 7.9, 10 μmoles $(NH_4)_2SO_4$, 20 μg of native rat liver DNA, 0.5 μmole $MnSO_4$ and RNA polymerase fraction in a final vol of 150 μl. After 60 min of incubation at 37°, RNA synthesis was measured as incorporation into $HClO_4$ precipitable material and counted on filter paper discs as previously described [11].

Protein was determined according to Lowry et al. [12].

3. Results

Using the procedures described above, the extraction and separation of RNA polymerase activity into different fractions on columns of DEAE-cellulose could be attained as shown in fig. 1. Two main peaks of RNA polymerase activity were found: one eluting at approx. 0.20–0.22 M NH_4Cl (A_b) and a second one between 0.25 and 0.3 M NH_4Cl (B). Peak A_b activity is resistant to the action of α-amanitin whereas activity of peak B is inhibited by this toxin. By increasing the duration of the exposure of the larval extract to 3.5 M ammonium sulfate from 3 to 12–14 hr

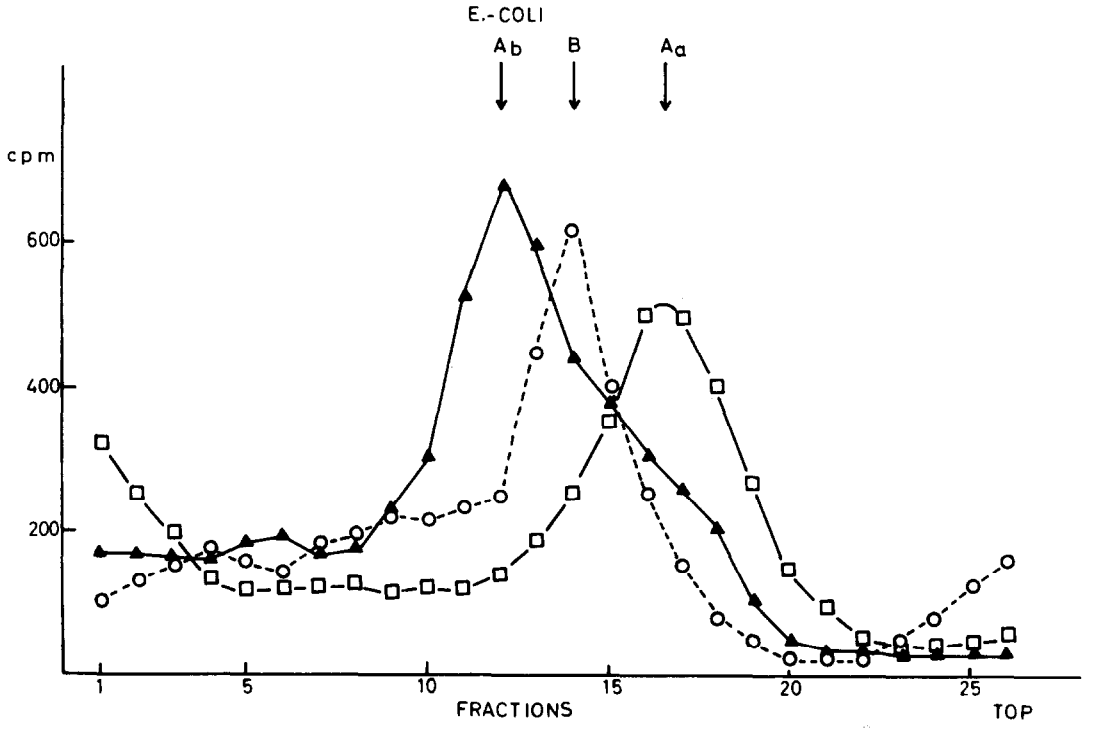

Fig. 3. Sucrose gradient centrifugation of polymerase forms A_a, A_b and B. 15 hr centrifugation at 36,000 rpm in a Beckman SW 41 rotor. 75 µl aliquots of each fraction have been tested in the assay described in methods. (□—□—□): polymerase form A_a, (▲—▲—▲): polymerase form A_b; (○ - - ○ - - ○): polymerase form B.

we could observe the appearance of a new peak of RNA polymerase activity eluting at 0.10–0.15 M NH_4Cl (fig. 2), insensitive to α-amanitin, which was either absent or faintly represented in the short extraction procedure. The peak fractions of the A_a, A_b and B components were dialyzed against medium A devoid of glycerol for 1 hr and then submitted to sucrose gradient centrifugation as described in methods. A_b and B show a higher sedimentation coefficient than A_a (see fig. 3). The ionic dependence of transcription by A_a, A_b and B are shown in fig. 4. All 3 forms of RNA polymerase are maximally stimulated by Mn^{2+}. Polymerases A_a and A_b are progressively inhibited by increasing the ionic strength whereas polymerase B shows maximal activity at ionic strength 0.2.

The effects of different templates on transcription is shown in table 1. Polymerase B transcribes heat denatured DNA 3–4 times as effectively as native DNA. Polymerase A_a and A_b also transcribe more efficiently heat denatured than native DNA, but not at the high rate of polymerase B. Rat liver DNA is transcribed more efficiently than calf thymus DNA whereas T4 DNA is transcribed very poorly.

The effects of different metabolites on the RNA polymerases is shown in table 2 and in fig. 5. α-Amanitin inhibits exclusively polymerase B, the doses for the inhibition being higher than those needed to affect rat liver polymerase B (fig. 5). The different forms of RNA polymerase are resistant towards rifamycin. AF-05 and AF-13 and Lucanthron in very high concentrations inhibit all 3 RNA polymerases whereas Lasiocarpin shows only a very slight inhibitory action (table 2).

4. Discussion

RNA polymerase from the integument of blowfly

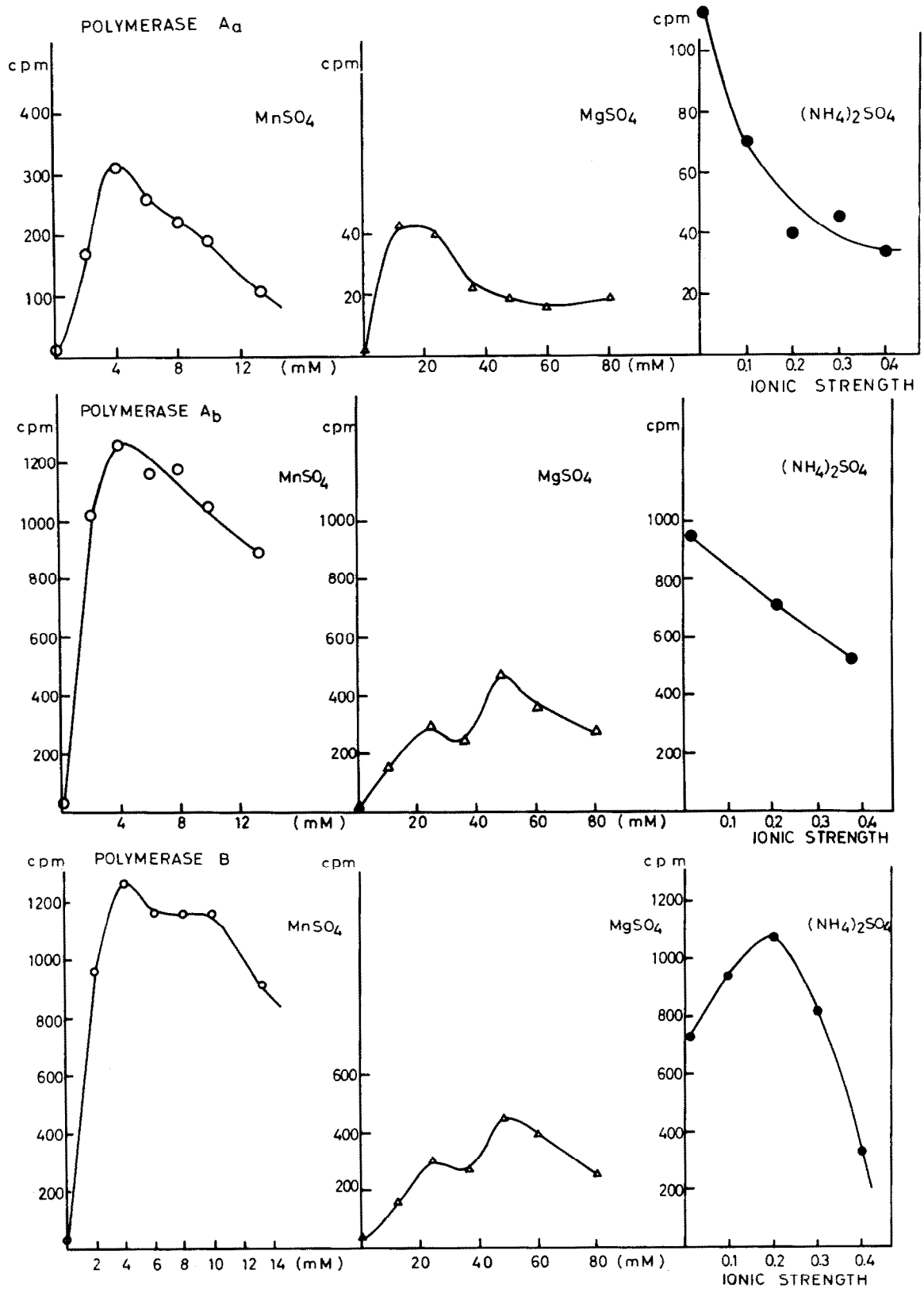

Fig. 4 a–c. RNA polymerase activity as a function of differing concentrations of Mn^{2+} or Mg^{2+} and effect of ionic strength on the rate of transcription, tested in the presence of Mn^{2+}-ions in the optimal concentration. The cpm values represent acid-precipitable material in 100 µl aliquots of the incubation mixture (150 µl) as described in methods.

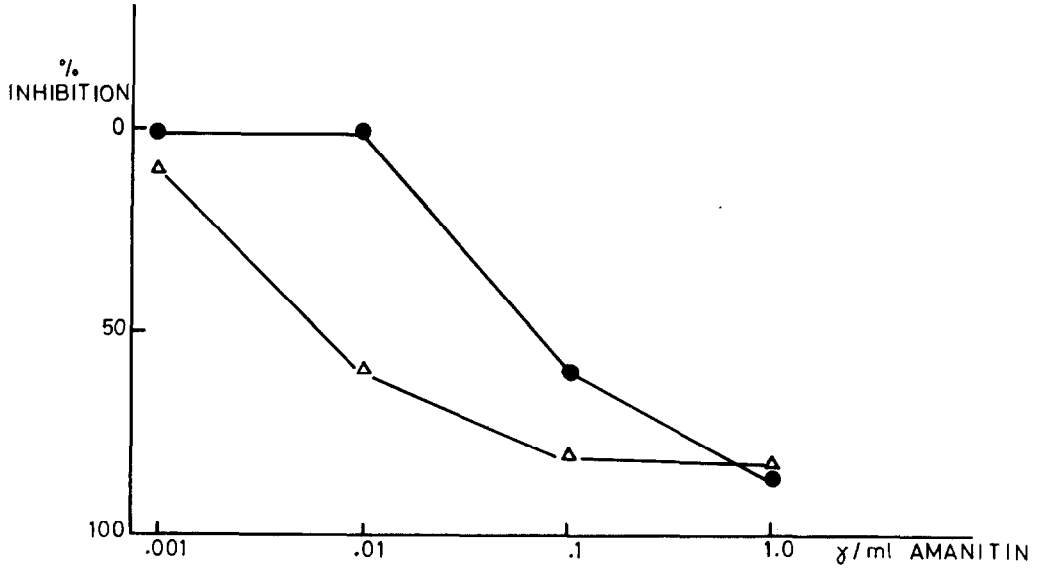

Fig. 5. Inhibitory effect of α-amanitin on transcription by RNA-polymerase B from insect (●—●—●) and rat liver (△—△—△).

larvae exists in multiple forms as already described for a variety of other species. Three main activities can be demonstrated. One, polymerase B, elutes from a DEAE-cellulose column at 0.3 M NH_4Cl, is α-amanitin sensitive, transcribes heat-denatured DNA much more efficiently than native DNA and shows maximal activity at ionic strength 0.2. The other 2 activities, A, eluting at 0.1–0.15 M NH_4Cl, and A_b, eluting at 0.20–0.22 M NH_4Cl are insensitive to α-amanitin, are inhibited at high ionic strength and transcribe single stranded DNA better than native DNA. This is in contrast to the results obtained with A enzymes from other species which transcribe predominantly double stranded DNA. Polymerase A_a appears in significant amounts in preparations which have been exposed to ammonium sulfate for many hours. This raises the possibility that polymerase A_a arises from polymerase A_b either due to degradation of the enzyme or to dissociation of a subunit. This is supported by the results of the sucrose gradient centrifugation which shows polymerase A_a being of a smaller molecular size than polymerase A_b.

All 3 forms are maximally stimulated with Mn^{2+}. As well known, mammalian polymerase A is stimulated equally well by Mg^{2+} and by Mn^{2+}. Enzyme B, as all other B enzymes from a variety of sources, is sensitive to α-amanitin, but requires a much higher dose of the toxin for inhibition than the rat liver B enzyme. The same holds for the action of the rifamycin derivatives, which, at very high doses, inhibit RNA polymerase action.

The use of the integument of blowfly larvae as starting material for the present study, parallel to the relevance for comparative reasons, is based on the fact that it is a target tissue for the action of the moulting hormone ecdysone [13]. Ecdysone stimulates RNA synthesis and induces enzyme formation in the integument in a very defined period of larval life [14] and it will be interesting to assess whether these processes could be correlated with changes in the amount or patterns of RNA polymerase as suggested for other hormonally stimulated systems [15, 16].

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