RESISTANCE OF HEPATIC RNA POLYMERASES TO COMPOUNDS EFFECTING RNA AND PROTEIN SYNTHESIS *IN VIVO*

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1. Introduction

Alterations in RNA synthesis of eukaryotic cells, in particular that of the ribosomal precursor species, after cycloheximide [1–9] or hormone treatment [10–21] have been described by several authors. In order to understand the molecular basis for these changes, it is very important to assess whether the amount of RNA polymerase is responsible for these variations, suggesting a regulatory function for the concentration of RNA polymerase in these systems.

Recently it was reported that cycloheximide [22] and cortisol [23] administration gave rise to significant variations of hepatic RNA polymerase activity and/or level. We present data in this report demonstrating that cycloheximide or cortisol treatment *in vivo* has no significant effect on the amount of RNA polymerase when isolated by the procedure described [24].

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2. Materials and methods

[^{3}H]UTP (13.6 Ci/mmole) and L-[^{14}C] tyrosine (30 Ci/mmole) were obtained from the Radiochemical Centre (Amersham), nucleoside triphosphates, creatine phosphate and creatine phosphokinase from Boehringer (Mannheim); DEAE-cellulose (DE 32; 1 meq/g) from Whatman (London). Cortisol was a gift of Schering AG (Berlin), cycloheximide was purchased from Serva (Heidelberg). All other chemicals were reagent grade from Merck (Darmstadt).

In the case of the cycloheximide experiments, groups of three male Wistar BR II rats (120–140 g) were injected intraperitoneally either with saline or cycloheximide (0.4 mg/100 g body weight) at 0 time. At times varying from 5 min (only allowing resorption of the drug) to 4, 12 and 24 hr, a control group and

Table 1
Inhibition of hepatic protein biosynthesis by cycloheximide *in vivo*.

Group:	K_0	C_0	K_4	C_4	K_{12}	C_{12}	K_{24}	C_{24}
Cycloheximide:	–	+	–	+	–	+	–	+
Time* (hr):	0	0	4	4	12	12	24	24
Protein (cpm/mg):	2105	2097	3338	437	2884	691	3365	2230
Inhibition (%):		0.5		86.9		76.0		33.7

* Animals were killed at the indicated times after intraperitoneal injection of the drug (400 μg/100 g body weight). 0 hr implies that rats were sacrificed after 5 min, only allowing resorption of the inhibitor. Protein was determined by the Lowry method. Radioactivity was determined in the ultracentrifuged cytoplasmic fraction by precipitation with 1.1 M trichloroacetic acid. Pellets were rewashed 3 times with 12% TCA, 2 times with methanol, dissolved in formic acid, and counted in 15 ml Bray's solution [25].

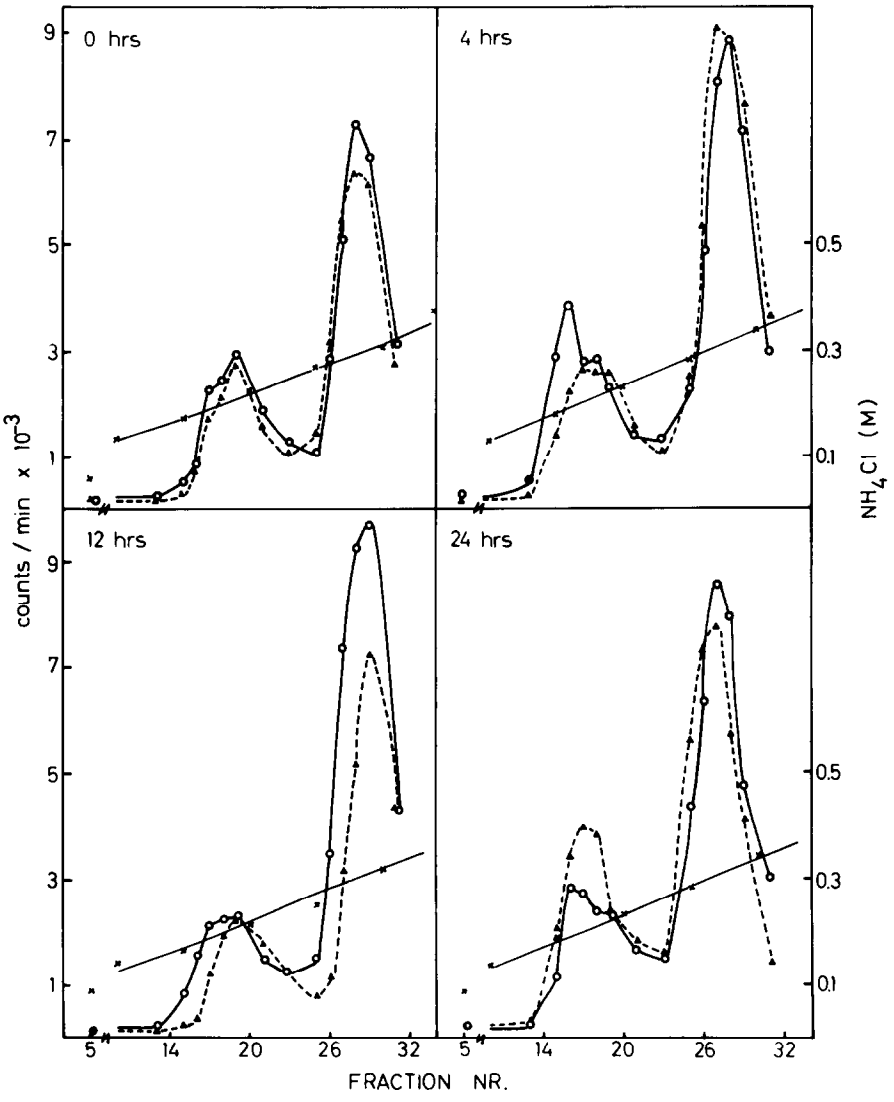

Fig. 1. Simultaneous chromatography of nuclear RNA polymerase activities from control (▲--▲--▲) and cycloheximide treated (o—o—o) animals on identical 2.5 X 5 cm DEAE-cellulose columns. Elution of approx. 3.5 ml fractions was achieved with a linear gradient from 0.0 to 0.35 M NH_4Cl in buffer (Tris-Cl, 0.05 M, pH 7.9, 20% glycerol, 5 mM mercaptoethanol, 0.25 mM EDTA) pumped from an ultrograd mixer at a flow rate of 40 ml/hr. In order to achieve identical conditions all columns were packed from the same batch of exchanger and were developed in pairs at identical elution rates from one pump (with multiple outlets) from the same elution medium.

a cycloheximide treated group were killed simultaneously. One animal of each control and inhibitor treated group received L-[^{14}C]tyrosine intraperitoneally (22 μCi/100 g body weight) 30 min before sacrifice, thus monitoring the extent of protein bio-synthesis as outlined in table 1. In the hormone trials, rats were adrenalectomized 3 days prior to the experiment. Two groups of eight animals were each injected intraperitoneally with saline or cortisol (3 mg/100 g body weight) 3 hr before sacrifice. Nuclear RNA polymerases A and B and cytoplasmic RNA polymerase C were extracted from the liver and measured *in vitro* as described by Seifart et al. [24].

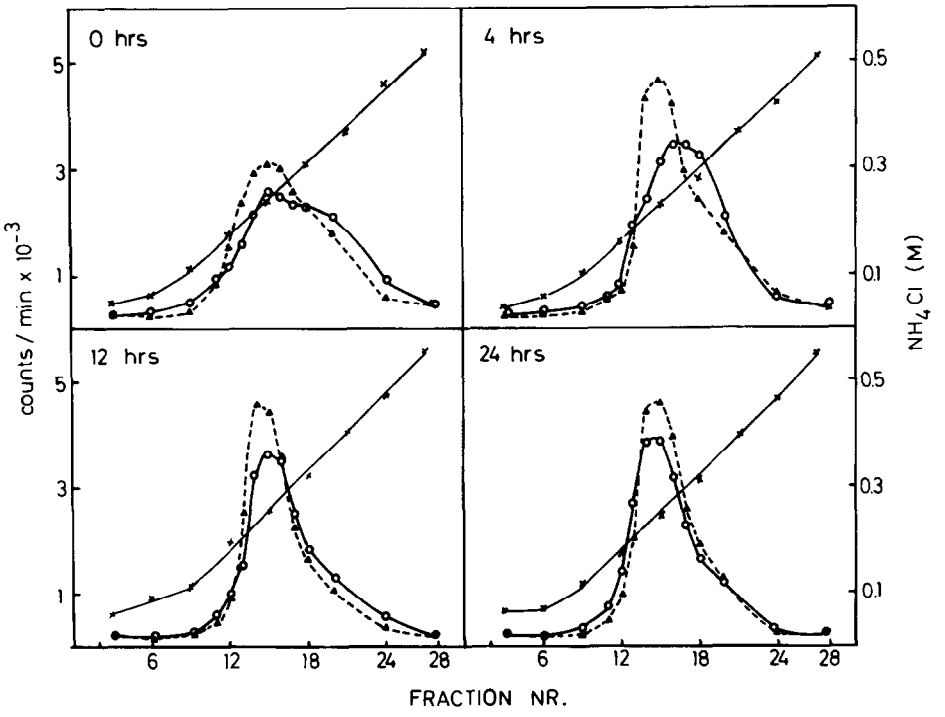

Fig. 2. Simultaneous chromatography of the cytosol from control (▲--▲--▲) and cycloheximide treated (○—○—○) animals on identical 2.5 X 5 cm DEAE-cellulose columns. Conditions were as described in fig. 1. In this case the developing gradient was from 0.0 to 0.60 M NH_4Cl.

3. Results

3.1. *Effect of cycloheximide on RNA polymerase activity*

In order to obtain an estimate of the half-life of hepatic RNA polymerase species from rat liver, cycloheximide was administered *in vivo* for several time periods ranging from 5 min to 24 hr. All steps of extraction and particularly purification of the enzymes from inhibitor-treated and the corresponding control groups were conducted simultaneously under identical conditions, as described in fig. 1, thus ensuring that the corresponding curves of enzyme activities are directly comparable.

Fig. 1 depicts the profiles obtained upon DEAE-cellulose chromatography of nuclear RNA polymerase activities A and B extracted after the indicated times of cycloheximide injection. The values in the figures represent absolute activities expressed as counts per minute. Correction for protein content has deliberately not been undertaken since protein concentrations could vary as a function of cycloheximide treatment and a correction for this variable could then possibly eliminate an effect which the experiment was designed to measure. However, extreme care was taken to equalize experimental conditions as outlined, thus allowing a direct comparison of individual column profiles.

When comparing RNA polymerase activities of control and cycloheximide treated animals, no marked difference can be detected for either nuclear enzyme A or B. This applies even to the longest times of cycloheximide application and to the ratio of the two enzymes A/B within any one column. The apparent activity of the A enzyme is lower in all cases which is a function of the assay conditions (Mn^{2+}; $(NH_4)_2SO_4$; heat denatured DNA) favouring enzyme B. Differences which do occur between treatment-groups do not follow the time of cycloheximide application and could be attributed to experimental error.

The results obtained for the enzyme extracted from the cytoplasm (fig. 2) likewise convey the con-

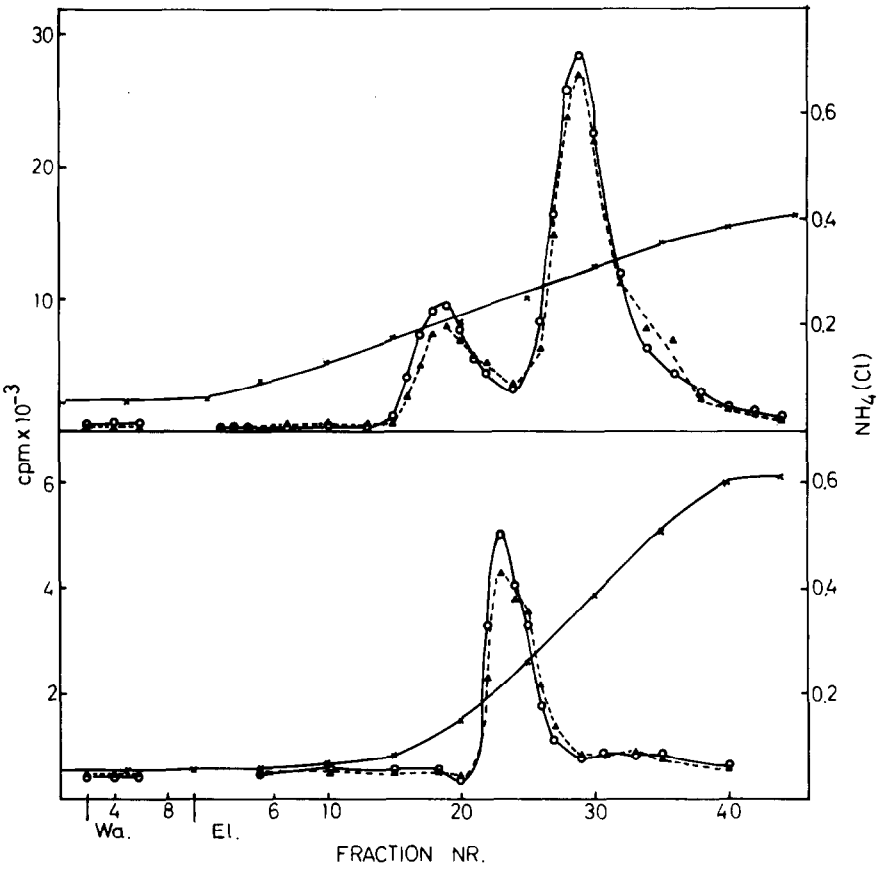

Fig. 3. Simultaneous chromatography on identical 2.6 × 13 cm DEAE-cellulose columns of RNA polymerase activities isolated from nuclei (part A) or cytoplasm (part B) from adrenalectomized animals treated with saline (control ▲--▲--▲) or cortisol (o—o—o). Conditions were as described in fig. 1 and 2.

clusion, that there is no significant decrease in enzyme activity during a 24 hr period after cycloheximide treatment.

Table 1 shows the degree of inhibition of overall protein synthesis measured concomitantly by [^{14}C]tyrosine incorporation as described in Methods. It clearly shows a drastic inhibition of protein synthesis (± 86%) 4 hr after administration of the drug which was still 76% after 12 hr. At 24 hr protein biosynthesis was restored to 66% which was also reflected in a recovery of the general health condition of the animals. It was not attempted to achieve complete inhibition of protein synthesis, since pilot experiments had demonstrated that higher dosages of cycloheximide totally suppressing amino acid incorporation (8 mg/100 g body weight) killed all the animals within 6 hr, thus preventing an assessment of slowly turning over proteins.

3.2. *Effect of cortisol on RNA polymerase activity*

Fig. 3 represents the profile on DEAE-cellulose of RNA polymerase isolated from nuclei (part A) or cytoplasm (part B) from adrenalectomized animals treated with saline (controls) or cortisol. The columns containing the nuclear extract were extensively washed with buffer into individual fractions as indicated in fig. 3, in order to detect a polymerase species designated as 1 A by Sajdel et al. [23] and also reported by Schmuckler et al. [20]. Our data show only two RNA polymerase activities eluting from this column at 0.19 M and 0.30 M NH_4Cl, corresponding to RNA polymerase A and B [26, 24]. Apart from

insignificantly lower values for the control curve related to a lower protein concentration, there is no marked difference between treatment-groups. Virtually the same results are obtained if polymerase C is isolated from either adrenalectomized or hormone treated animals and analyzed on DEAE-cellulose columns (fig. 3B).

4. Discussion

The data presented here lead to the conclusion that the amount of nuclear RNA polymerase A and B, as well as of cytoplasmic enzyme C isolated from rat liver tissue by the technique described [24], remain unaltered after a 24 hr treatment period of cycloheximide or 3 hr application of cortisol *in vivo*. These findings are in disagreement with results reported by Yu and Feigelson [22] who found a rapid decrease of nucleolar RNA polymerase activity in rat liver after cycloheximide application, measured as UTP incorporation of intact nucleoli under certain experimental conditions. These workers concluded from their findings that polymerase A has a rapid turnover time and that control of RNA synthesis, in particular of ribosomal RNA, can effectively be achieved by a variation in the amount of enzyme synthesized. This assumption would be reconcilable with experiments stating an enhanced amount of nucleolar RNA polymerase after hormone treatment [20, 23, 27]. This conclusion is, however, not supported by data presented here and results of Sekeris and coworkers [21, 28].

All the RNA polymerase species from rat liver studied here seem to be unaffected by conditions which lead to a strong inhibition of protein synthesis for at least 12 hr. It is also interesting to note, that at a time when general protein synthesis is in the recovery phase (24 hr; table 1), there is no drastic change in the amount of RNA polymerase, in the sense of a rebound effect. Therefore the enzyme molecules seem to have extended half life periods, representing a fairly stable population. Among others, one of the aims of these experiments was to possibly determine the biosynthetic interrelationships between enzymes A, B and particularly C. It is questionable however, whether this approach is feasible with proteins of very long half-life values. The conclusions drawn from these experiments are valid under the assumption that the inhibition of protein synthesis by cycloheximide is random for all proteins and that the level of residual protein synthesis (± 10%) required to keep the animals alive for periods over 6 hr, does not account for completely normal synthesis of RNA polymerase. No such selective mechanism of escape-synthesis after cycloheximide has been described, however, and experiments conducted with 3.0 mg cycloheximide/100 g for 4 hr showed a complete blockade of peptide synthesis but maintained enzyme levels exceeding 85% of the control values.

The remarkable inertia of the RNA synthesizing enzyme must be viewed in the light of the well documented inhibition of RNA synthesis by cycloheximide [1–9]. It is therefore rather difficult to assume that the rate of RNA synthesis can be controlled through enzyme concentrations. In contrast, the situation seems to apply that RNA polymerase may be present in large excess, and the presence of a cytoplasmic enzyme [24] could be an expression of this abundance. Recent calculations by Chambon [29] show an average number of 3.9×10^4 polymerase B molecules per haploid genome, containing a tentative number of 50,000 promotor sites. If it is assumed that only a portion of these are active in native chromatin, these calculations would support the conclusion concerning the relative abundance of the RNA polymerase molecule in relation to the number of specific initiation points of RNA synthesis. Therefore regulation of RNA synthesis in the eukaryotic nucleus is possibly achieved through other factors controlling the rate of this process. It is possible that the multiple forms of RNA polymerase recognize specific and different promotor sites [30, 31]. This question is, however, related to specificity and not rate of RNA synthesis.

It is also of importance to note, that synthesis of ribosomal and of heterogeneous nuclear RNA are possibly interrelated as has recently been concluded by Schmid and Sekeris [32]. It is possible that shortlived proteins, as have been postulated by Muramatsu [7] which could be synthesized on short-lived RNA messages, function as such specifiers of RNA synthesis.

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