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SITE OF ACTION OF COLCHICINE ON RNA RELEASE FROM LIVER NUCLEI

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SUMMARY Colchicine inhibition of messenger RNA release from rat liver nuclei has been analyzed in a cell-free system, which supports the processing and transport of functional RNA. The degree of inhibition is porportional to the colchicine concentration above 300 μM. The site of action is not microtubular elements associated with the nuclei, since some colchicine analogs containing a trimethoxy-benzene ring do not disrupt microtubules, but still inhibit RNA transport. Furthermore, the Vinca alkaloids, which like colchicine disrupt microtubules, do not inhibit messenger RNA transport. From an assessment of its effect on nuclear RNA metabolism, it is concluded that colchicine inhibits RNA release by inhibiting RNA transport rather than RNA processing. This effect may be due to the specific binding of colchicine to the nuclear membrane.

A reconstituted cell-free system has been described (1-3) which supports the release from isolated nuclei of functional messenger RNA. This system requires both energy (ATP) and a specific class of cytosol proteins. It has proven to be useful for studying the regulation of messenger RNA processing and transport and in particular, nucleocytoplasmic controls. The ATP-requirement may be related to phosphorylation of the cytosolic transport factors (4), the activity of a nuclear pore nucleoside triphosphatase, (5), or both. The cytosolic protein factors essential for transport may interact directly with the transported RNA (6). In addition to specific interaction of hormones with the nuclear membrane (7), other substances, or conditions have been identified which modify the nuclear membrane to promote or inhibit nuclear release of RNA. These include temperature (8) and beryllium or strontium (9).

We have previously shown (10) that colchicine has a marked inhibiting effect on messenger RNA transport from rat liver nuclei. This observation has

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been confirmed (11). Although the site of action of colchicine has not been determined, it could effect either nuclear RNA processing, or transport. The most probable site of action is at nuclear-cytoplasmic RNA transport through the nuclear pores, since colchicine is known to interact with both microtubules (12) and with plasma (13) and nuclear (14) membranes. The purpose of the present investigation was to identify the site of action of this alkaloid, since it might be of use in elucidating cellular controls.

MATERIALS AND METHODS

Animals: Subcellular fractions were prepared from the livers of 250gm male Sprague Dawley rats which were fasted 16 hrs prior to use.

Reconstituted Cell-free system: Nuclear RNA was prelabeled in vivo with [6-^{14}C]orotic acid (40μCi/250 gm, i.p.) for 30 min before removal of the liver. In specified experiments, the rats were pre-treated for 15 min with colchicine (10 mg/250gm, i.p.) before administering the labeled RNA precursor. Nuclei were isolated after homogenizing the liver in hypertonic sucrose as previously described (1-3). The cytosol fraction was prepared separately (1-3) and dialyzed overnight before use.

The cell-free system consisted of prelabeled nuclei ($5x10^6$/ml) in medium designed to provide transport proteins, preserve the nuclear structure and to suppress nuclease activity. The medium contained, 50 mM Tris-HCl (pH 7.5), 2.5 mM $MgCl_2$, 25mM KCl, 0.3mM $CaCl_2$, 0.5 mM $MnCl_2$, 2.5 mM Na_2HPO_4, 5.0 mM NaCl, 5.0mM spermidine, 2.5mM phosphoenol- pyruvate, 6.4 units of pyruvate kinase per ml., dialyzed cytosol (12.0 mg protein/ml) and 0.3 mg/ml of low molecular weight yeast RNA. Unless indicated otherwise, the incubation was performed at 30°C for 30 min.

Analysis of Transported RNA: The transported labeled RNA was precipitated from the post-nuclear supernatant of the assay with 0.1 vol. 50% trichloroacetic acid at 0°C. After extraction with ethanol the pellet was radioassayed in liquid scintillant. Alternatively, the RNA was purified from the nuclei-freed incubation medium with phenol:chloroform (15), prior to size distribution analysis on 10-30% sucrose gradients, or estimation of the poly(A) content on an oligo(dT) column (16).

Analysis of Nuclear RNA: Nuclei ($3x10^6$) from 6.0 ml of incubation medium were homogenized in 0.14 M NaCl-0.5M sodium acetate buffer, pH5.1, containing 0.5% sodium dodecyl sulfate (1.5 ml/gm liver, 4°C). The RNA was deproteinized with an equal volume of acetate-buffer-saturated phenol-0.1% -hydroxy-quinoline at 65°C for 10 min, then at 25°C for 15 min. After separating the phases, the aqueous phase was re-extracted twice with fresh phenol at 25°C. After precipitation with 2.5 vols. 95% ethanol, the RNA was analyzed on 10-30% sucrose gradient (Beckman SW25.1 rotor) with a 5.0 ml 2.0 M sucrose cushion. The resuspension and gradient buffer was 0.1 M NaCl-20mM sodium acetate-1.0mM ethylenediamine tetracetate, pH 5.0. 18S and 28S ribosomal RNA were included as markers in the layered sample. The gradients were centrifuged (16 hrs at 51,000 g. av), then scanned (260 nm) and 1.0 ml fractions were radioassayed in liquid scintillant.

Further details of the in vitro-transport system and the analysis of transported and residual nuclear RNA are given in previous publications (1-3, 7, 10, 16, 17).

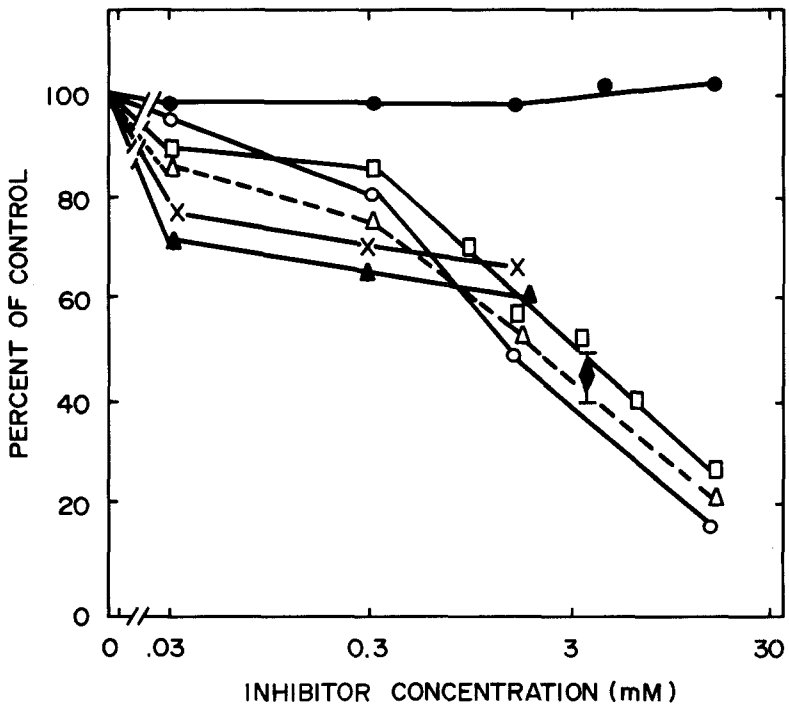

Fig. 1. The effect of alkaloids and related compounds on RNA release from isolated nuclei in the cell-free system. Nuclei, prelabelled in vivo for 30 min in the presence of 150 µg/kg body weigh of actimonycin D to inhibit labeling of ribosomal RNA, were preincubated in the absence of ATP and the energy regenerating system, but with the indicated concentrations of colchicine, or the specified compounds. Following the pre-incubation at 0°C for 15 min and the addition of ATP and the energy regenerating system, incubation was continued for 20 min at 30°C. The maximum RNA transported in the contol system (no inhibitor) was approximately 6% of the total nuclear counts during the 20 min incubation. These and subsequent experimental data represent the average of 2 experiments which duplicated to within 10%. Shown are the concentration dependence curves for inhibition of RNA transport by colchicine (□—□), lumicolchicine (△—△), podophyllotoxin (○—○), 3,4,5-trimethoxy-benzyl alcohol (X—X), 3,4,5-trimethoxycinnamic acid (▲—▲), and Vinblastine,(●—●) (Vincristine gave identical results). Also shown (one point: ◆) is the lack of effect of washing the nuclei after the pre-incubation with 3.3 mM colchicine by sedimenting them through 1.0M sucrose, on the inhibition of RNA transport.

Preparation of Lumicolchicine: This analog was produced from colchicine by U.V. irradiation (18) and its purity was confirmed by absorption spectrum and melting point analysis.

RESULTS AND DISCUSSION

Initial in vivo experiments (unpublished data) demonstrated a 45% decrease in labeled RNA release from liver nuclei to cytoplasm when nuclear RNA was prelabeled for 15 min with [6-^{14}C]orotic acid before the administration of colchicine (0.025 m moles/kg body weight) and 30 min before removal of the liver. Further, the primary site of action of the alkaloid

appeared to be post-transcrptional i.e. on nuclear RNA processing, or nucleocytoplasmic transport, since the net effect of colchicine was not reduced by pretreating the experimental and control animals with actinomycin D after 30 min prelabeling period to inhibit further transcription.

The effect of colchicine, its analogs and Vinblastine (Vincristine) on post-transcriptional nuclear events was studied further in a reconstituted cell-free system. Dose-response curves for the inhibition of nuclear RNA release by these compounds are shown in Fig 1. The protocol involved pre-incubation of the 30 min pre-labeled nuclei for 15 min at 0-°C in the specified medium of the cell-free system (cf. Materials and Methods) minus the energy sources, but in the presence of the indicated concentrations of the specified compound. RNA transport was then assayed by incubating for 20 min at 30°C in the presence of energy. All of the compounds except vinblastine (and vincristine) inhibited RNA release. Colchicine contains both a trimethoxy-benzene and tropolone ring. Lumi-colchicine, which lacks the tropolone ring, also inhibits RNA release, but unlike colchicine does not bind to microtubular elements (19). Podophyllotoxin, which also lacks the tropolone ring but binds to microtubules (cf. 19) also inhibits RNA release. Since all of these compounds have in common the trimethoxybenzene ring, it is not surprising that 3,4,5-benzyl alcohol and 3,4,5-trimethoxycinnamic acid also show significant activity. 3,4,5-Trimethoxybenzoic acid and 3(3,4-dimethoxyphenyl) propionic acid showed only very slight activity (results not shown). Thus, the trimethoxy-benzene ring appears to be an essential component for inhibition, but other structural features are obviously also important.

The data in Fig 1. indicate that the binding of colchicine to the nuclei is relatively strong. Thus, following incubation of the nuclei at 0°C with 3.3 mM colchicine, the inhibition of RNA release could not be reversed by purifying the nuclei through a layer of 1.0 M sucrose (3,000 xg for 15 min). Furthermore, this experiment confirms that the inhibitory action of colchicine is mediated by direct interaction with the nucleus.

TABLE I
Specificity of colchicine binding to nuclei *in vitro*

Unlabeled colchicine(mM)	[^{3}H]colchicine bound (% of control)
0	100
2.5	73
5.0	42
12.5	29
25.0	6

Liver nuclei (5.5 x 10^6/ml) were incubated in the cell-free system minus energy, but with [^{3}H] colchicine (10μCi/ml) and the indicated amounts of unlabeled colchicine, for 15 min at 0°C. The nuclei were then recovered by centrifugation, extensively washed and radioassayed after solubilization.

The interaction of colchicine with the nuclear membrane appears to be relatively specific as a 100-fold excess of unlabeled colchicine virtually eliminated the binding of the [^{3}H] colchicine (Table 1). Essentially no binding was observed when the nuclear membrane was removed with detergent (NP-40).

Theoretically colchicine might inhibit RNA release by interfering with intranuclear RNA processing, nucleo-cytoplasmic RNA transport, or both. To identify the molecular site of action of the alkaloid, the effect of colchicine on the nuclear processing of premessenger RNA was investigated. For this analysis, the liver RNA was prelabeled *in vivo* with [^{14}C]orotic acid (30 min) in the presence of 150μg/kg body weight of actimonycin D, a dose just sufficient to inhibit nucleolar preribosomal RNA synthesis, without interfering significantly with messenger RNA transcription (20). The isolated nuclei were then incubated in fortified homologous cytosol in the absence (control), or presence (experimental) of 3.3 mM colchicine. The control and experimental nuclei were pre-incubated for 15 min. at 0°C before assay to insure maximal inhibition of RNA release by colchicine during the subsequent 30 min RNA release assay at 30°C. The initial size distribution of the nuclear RNA was determined on nuclei held at 0°C for 45 min in the presence, or absence of colchicine. Folowing phenol:chloroform extraction of the RNA the size distribution was determined on 10-30% sucrose gradients with a

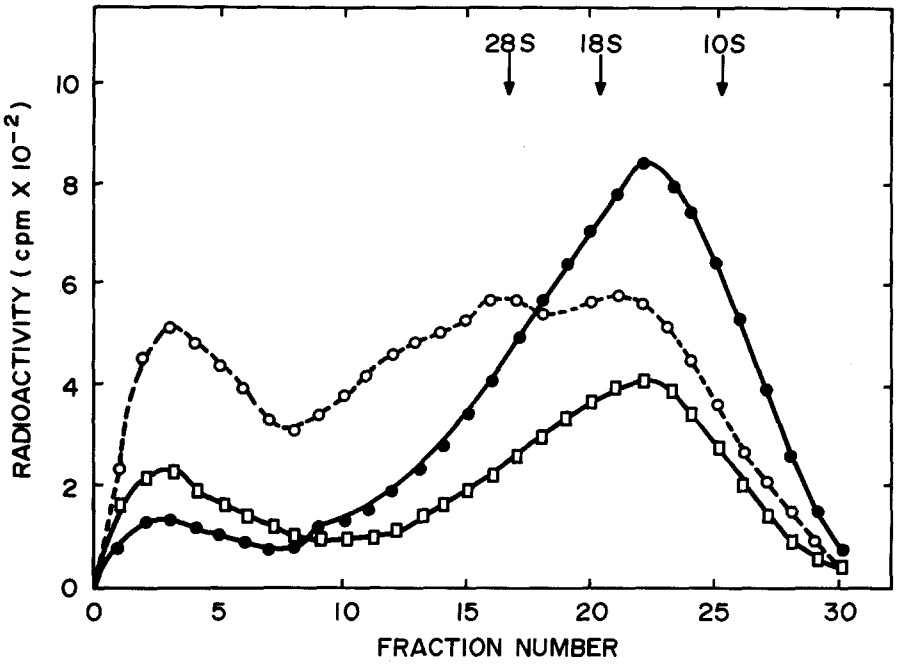

Fig. 2. Size distribution profile of labeled nuclear RNA after a 30 min *in vitro* incubation at 0°C (O—O), or at 30°C in the presence (●—●), or absence (□—□) of 3.34mM colchicine. The nuclear RNA was prelabeled *in vivo* in the presence of 150µg/kg of actinomycin D, then pre-incubated at 0°C for 15 min with (or without) colchicine, before carrying out the RNA transport at 30°C. The purified nuclear RNA was analyzed on sucrose gradients. Since the profiles of nuclear RNA incubated at 0°C in the presence and absence of colchicine were identical, only the latter is shown. Other details of the pre-incubation and transport assays are given in Materials and Methods and Fig. 1.

cushion of 2.0M sucrose at the bottom. The size distribution of the labeled RNA (mostly pre-messenger RNA) in the control and experimental nuclei are shown in Fig. 2. The data indicate that colchicine does not detectably inhibit the intranuclear processing of pre-messenger RNA. Thus the efficiency of conversion of the high molecular weight to the low molecular weight form appears to be slightly greater, rather than less, in the presence of colchicine. This effect is opposite to that observed with proflavine (17), an inhibitor of RNA processing. The higher concentration of the processed RNA in the nuclei incubated at 30°C in the presence of colchicine as compared to the control (no colchicine) is explained by the fact that RNA transport is much more limiting in the presence of the alkaloid. The average size distribution of the processed messenger RNA in the nucleus (i.e. 18S) is similar to that of the RNA transported to the cytosol *in vitro* (16,21).

In summary, colchicine and certain analogs, thereof, inhibit the nuclear release of messenger RNA in vivo and in vitro. An analysis of this phenomenon indicates that colchicine interacts with the nuclear membrane to primarily affect transport rather than intranuclear processing; this inhibitory effect is not, however, mediated by the disruption of microtubular elements. Further, since the in vitro system utilizes an external energy source, the alkaloids inhibition is not mediated in vivo by effects on the energy charge. Also, colchicine alone, is capable of causing the inhibition, since the cell-free system is devoid of the liver microsome oxidase system known to convert this drug to a number of demethoxy and other oxidative derivatives (22). Finally the results are consistent with earlier autoradiographic studies (14,24) which indicated that colchicine locallizes at the nuclear membrane, in addition to microtubules in the intact cell, and histological evidence (25) that it interacts with the nuclear pore. The availability of a compound which inhibits RNA transport without affecting RNA processing in the in vitro system derived from rat liver should facilitate the investigation of these two nuclear processes.

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