Inhibition of RNA Synthesis in Ehrlich Tumor Cells by the Dialdehyde Derivative of Inosine (NSC 118994)¹

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ABSTRACT

The dialdehyde derivative of inosine, INOX (NSC 118994), was studied for its effect on RNA synthesis in Ehrlich tumor cells. INOX inhibited the incorporation of [¹⁴C]uridine into the RNA of intact tumor cells. Polyacrylamide gel electrophoresis and sucrose gradient centrifugation analysis of the RNA showed that the synthesis of preribosomal RNA and high-molecular-weight RNA was inhibited by INOX. The dialdehyde derivatives of adenosine 5'-triphosphate and inosine 5'-triphosphate inhibited RNA synthesis in isolated nuclei. The dialdehyde derivatives of 5'-inosinic acid and inosine when they were added to isolated nuclei did not inhibit RNA synthesis. However, when tumor cells were incubated with INOX or the dialdehyde derivative of 5'-inosinic acid and the nuclei were then isolated, there was a marked decrease in RNA synthesis. This inhibition was both time dependent and concentration dependent. The inhibition of RNA synthesis in the nuclei from treated cells did not reverse by changing the culture medium. α-Amanitin and actinomycin D inhibited RNA polymerase activities in the nuclei from control cells to the same extent that they inhibited the residual activity in the nuclei from the treated cells. Nuclear extracts prepared from the INOX-treated cells did not show a decrease in RNA polymerase activity, indicating a reversal of the inhibition. The inhibition of RNA synthesis in the nuclei from INOX-treated cells was completely reversed by the addition of exogenous polydeoxyadenylate-deoxythymidylate as template, showing that the inhibition of RNA synthesis by INOX was not due to the inhibition of RNA polymerases but rather was due to chain termination of the growing RNA strand or impairment of template function.

INTRODUCTION

The dialdehyde derivatives of nucleosides have been shown to be relatively effective antitumor agents in selected animal systems (10). Kimball et al. (15), Nixon et al. (18), and Spoor et al. (22) have shown that the dialdehyde derivative of 6-methylthiopurine riboside reacted with certain enzymes in vitro to form Schiff bases, which resulted in the inhibition of the enzyme activity.

We have previously reported that the dialdehyde derivative of adenosine, AMP, and ATP inhibited ribonucleotide reductase activity in cell-free extracts (3, 4) and that adenosine and AMP dialdehyde derivatives inhibited nucleic acid synthesis in intact Ehrlich tumor cells in culture (6). The dialdehyde derivative of inosine, INOX (NSC 118994), has been started on Phase 1 clinical trials (13). Pharmacological and biochemical studies have shown that INOX is excreted in the urine as complexes of urine constituents and is found in the serum bound to protein (presumably through Schiff base interactions with NH₂ groups) (8). In addition, it was shown that INOX caused the cross linking of proteins (9).

Biological studies have shown that INOX had its greatest cytotoxic effects during the late G₂-early S phase of the cell cycle in Chinese hamster fibroblast cells (2). Other studies have shown that the inhibition occurred during G₂ (19). We have recently reported that INOX and PI-IMP inhibited ribonucleotide reductase activity and that both RNA and DNA synthesis were inhibited by these compounds (7).

It was to be expected that these dialdehyde derivatives of nucleosides would be quite reactive with primary amino groups and that nonspecific interactions with cellular constituents would occur. However, several studies have shown that the base moiety of the nucleoside dialdehyde derivative does influence the nature of the reaction, indicating that these nucleoside dialdehyde derivatives probably do have specific sites of interactions. For example, it has been shown that the dialdehyde derivative of 6-methythio-<br>purine riboside inhibited RNA polymerase (15, 21), whereas in other studies (9) it was shown that INOX had no effect on Escherichia coli RNA polymerase. In unpublished work from this laboratory, we have observed that the dialdehyde derivatives of purine riboside and guanosine were much less inhibitory to nucleic acid synthesis in Ehrlich tumor cells (J. G. Cory, unpublished results) than were the dialdehyde derivatives of adenosine (6) and inosine (7). Other studies have shown that L1210 cells were much more sensitive to INOX than were H.Ep.-2 cells. These studies further showed that there were quantitative differences between the effects of INOX and the dialdehyde of 6-methylthio-<br>purine riboside on L1210 cells (V. H. Bono, Jr., Drug Research and Development, Division of Cancer Treatment, National Cancer Institute, personal communication, from a report filed by Dr. L. Bennett and Dr. W. Brockman, Southern Research Institute.)

The inhibition of DNA synthesis by the dialdehyde derivatives of purine nucleosides correlated extremely well with the inhibition of ribonucleotide reductase activity (5); this

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then would appear to be 1 of the principal biochemical sites for the inhibition of DNA synthesis by these compounds. Studies with $[^{14}C]$INOX have indicated that $[^{14}C]$INOX was transported into Ehrlich tumor cells without cleavage of the N-glycosidic bond, and there was evidence that $[^{14}C]$INOX was phosphorylated and incorporated into RNA (11). In this report we present information on the mechanism of inhibition of RNA synthesis by the dialdehyde derivatives of inosine and IMP.

MATERIALS AND METHODS

Effects of INOX and PI-IMP on RNA Synthesis in Ehrlich Tumor Cells in Culture. The Ehrlich tumor cells were grown in female mice (ICR) by weekly inoculation of recipient mice with 0.2 ml of tumor cells as taken from the donor mice. The mice were purchased from Laboratory Supply Co., Indianapolis, Ind. The cell culture experiments were carried out under sterile conditions as previously described (6). The tumor cells were incubated in the presence and absence of inhibitor for 1.5 hr. $[^{14}C]$Uridine (392 mCi/mmmole; 1.25 µCi/flask) was added to each culture flask, and the incubation was carried out for an additional 30 min. The cells were collected by centrifugation. The RNA from the cell pellet was isolated by the method of Wilkinson and Pitot (25). Prior to further studies the RNA was put over Sephadex G-25 columns (25 x 2.5 cm) to remove any low-molecular-weight, UV-absorbing, 14C-labeled components. The RNA that eluted in the void volume was reprecipitated with 2 volumes of 95% ethanol containing 2% potassium acetate.

Electrophoresis of the RNA on polyacrylamide-agarose gel electrophoresis was carried out by the method of Wilkinson et al. (24). After electrophoresis the gels were scanned at 260 nm with a Beckman Model 25 spectrophotometer equipped with a gel-scanning module. The gels were then sliced into 2-mm sections and incubated overnight at 37° in 0.5 ml 0.5 N NaOH. HCl (0.5 ml; 0.5 N) was added to neutralize the NaOH. Scintillation fluid was added, and the samples were measured for radioactivity.

Sucrose gradient fractionation of the RNA was carried out on a linear sucrose gradient (5 to 47%) in sodium acetate buffer (20 mm; pH 5.1) containing 1 mm EDTA and 100 mm NaCl. The centrifugation was carried out in an SW 27 rotor for 16 hr. The gradients were fractionated with an Isco Model 680 gradient fractionator. The gradients were scanned for UV absorbance at 254 nm, and fractions were collected for radioactivity measurements.

RNA Polymerase Activity in Isolated Nuclei. Nuclei were isolated from Ehrlich tumor cells by the method of Muramatsu et al. (17). The reaction mixture for the RNA polymerase assay (1) contained in a final volume of 0.25 ml: 25 µmoles Tris-HCl, pH 8.1; 18.75 µmoles KCl; 2.5 µmoles mercaptoethanol; 1.25 µmoles magnesium acetate; 1.25 µmoles manganese chloride; 125 µmoles ammonium sulfate, pH 8.1; ATP, GTP, CTP, and UTP at 40 nmole each. The concentration of the labeled substrate that was added was 10 nmole in some experiments [3H]UTP (0.2 µCi/nmoles), $[^{14}C]$CTP (0.04 µCi/nmmole), or $[^{14}C]$ATP (0.04 µCi/nmmole) was used as the labeled nucleoside triphosphate; the nuclei added were 200 to 300 µg DNA. The reaction was carried out for 30 min at 37° and was stopped by the addition of 10% trichloroacetic acid (0.35 ml). The precipitate was collected by centrifugation. The pellet was resuspended and washed with 0.5 ml 5% trichloroacetic acid, collected by centrifugation, dissolved in 0.2 N NaOH (0.5 ml), and reprecipitated with 10% trichloroacetic acid (0.5 ml). The final pellet was dissolved in 0.2 N NaOH (0.1 ml), H2O (0.9 ml) was added, and the sample was transferred to scintillation vials. All RNA polymerase assays were carried out in triplicate. Heated controls served as blanks. The data were presented as cpm rendered acid insoluble per mg DNA in the nuclei.

RNA Polymerase Activity in Nuclear Extracts. The tumor cells were treated according to the method of Longacre and Rutter (16). The cells were disrupted in a French pressure cell at 14,000 psi. The broken-cell preparation was diluted with buffer to bring the ammonium sulfate concentration to 0.1 M. The suspension was centrifuged at 105,000 x g for 1 hr at 4°. The supernatant fluid was used as the nuclear extract. The reaction mixture contained in a final volume of 50 µl: 2.7 µmoles Tris-HCl, pH 7.9; 80 nmole MnCl2; 0.15 µmoles β-mercaptoethanol; 0.315 µmoles NaF; 1.2 nmole EDTA; 6 nmole dithioerythritol; 16 µg calf thymus DNA; 30 µmoles ATP, CTP, and GTP; 0.5 nmole [3H]UTP (1 µCi). The reactions were run for 10 min at 37° and were stopped by the addition of a mixture (0.15 ml) containing 0.04 M sodium pyrophosphate, 0.01 M EDTA, 3.55 A260 units denatured calf thymus DNA, and 0.4 mg bovine serum albumin per ml. The precipitate was collected on Whatman glass fiber filters. These assays were carried out in triplicate.

In Vitro RNA Synthesis in the Presence of Actinomycin D and Poly(dA-dT). The method of Herzog and Farber (12) was used to determine the activity of RNA polymerase in isolated nuclei using exogenous poly(dA-dT) template (12). The assay mixture contained in a final volume of 0.25 ml: 0.5 µmoles MnCl2; 2 µmoles KCl; 0.5 µmoles β-mercaptoethanol; 20 µmoles ammonium sulfate; 1.25 µmoles magnesium acetate; ATP, GTP, and CTP at 40 nmole each; 10 nmole [3H]UTP (2 µCi). Actinomycin D and poly(dA-dT) were added at final concentrations of 20 µg/ml and 2 A260 units, respectively. The reactions were carried out for 30 min at 37°. These assays were carried out in triplicate.

Preparation of Periodate-oxidized Inosine, IMP, ITP, and ATP. The periodate-oxidized derivatives of inosine, IMP, ITP, and ATP were prepared by the method of Khym and Cohn (14). INOX was isolated on a Dowex 1-formate column with 0.02 M formic acid as the eluting solvent. PI-IMP was separated on a Dowex 1-formate column with a linear gradient of 0.02 to 4.0 M formic acid to elute the PI-IMP. The preparation, purification, and identification of these compounds have been previously reported in detail (7). The PI-ITP and PI-ATP compounds were separated from the iodate by chromatography on Sephadex G-10 (100 x 2.5 cm) with water as the eluting solvent and also on DEAE-Sephadex A-50 with triethylammonium bicarbonate as the eluting solvent. The products were shown to be free of iodate or periodate by the sensitive starch-iodate test (23) and to contain aldehyde functional groups by the method of Zamecnik et al. (26). Preparation of $[^{14}C]$INOX. $[^{14}C]$INOX was prepared from
Inhibition of RNA Synthesis

RESULTS

Inhibition of RNA Synthesis by INOX. Previous studies (7) had shown that INOX inhibited the incorporation of \([^{14}C]\)cytidine into RNA as determined by fractionation of the cellular macromolecules by the procedure of Schmidt and Thannhauser (20). In the present studies total cellular RNA was isolated by phenol extraction (25), and the RNA was then separated by gel electrophoresis and sucrose gradient fractionation.

Chart 1 shows the effects of INOX on the labeling pattern of the RNA species as separated on 2.4% polyacrylamide gel electrophoresis. Under the conditions used for this experiment with 0.75 mM and 1.0 mM INOX, RNA synthesis was inhibited 28 and 47%, respectively. There was inhibition of the incorporation of \([^{14}C]\)uridine into the 45S ribosomal RNA precursor. The 4S and 5S RNA species moved off the gel under these conditions.

The same RNA samples were run on linear sucrose gradients (5 to 47%), and the gradients were fractionated. These data are shown in Chart 2. Analysis of the data in Chart 2 indicates that the synthesis of the 45S RNA and the other high-molecular-weight species was more effected than was that of the smaller RNA species.

In another set of experiments, Ehrlich tumor cells were incubated in culture with and without INOX (1 mM) for 1.5 hr and then were labeled with \([^{3}H]\)uridine for 30 min. The cells were collected, and the nuclei were isolated from these cells. The RNA was isolated from the nuclei and subjected to sucrose density centrifugation. Results of this experiment are shown in Chart 3. Again, a marked inhibition of RNA synthesis occurred in the presence of INOX.

Inhibition of RNA Synthesis in Isolated Nuclei. Nuclei were prepared from Ehrlich tumor cells. These nuclei were used to determine the effect of INOX, PI-IMP, and PI-ITP on the RNA polymerase activity. These data are shown in Table 1. INOX and PI-IMP did not inhibit the RNA synthesis in...
Table 1
Effect of dialdehyde derivatives of RNA synthesis in isolated nuclei

The nuclei were prepared from Ehrlich tumor cells as described in "Materials and Methods." The RNA polymerase assays were carried out in triplicate as described in "Materials and Methods," and the dialdehyde derivatives of inosine, IMP, ITP, and ATP were added to the isolated nuclei at the concentrations indicated.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Concentration (mM)</th>
<th>Labeled nucleoside triphosphate</th>
<th>% of control</th>
</tr>
</thead>
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<tr>
<td>INOX</td>
<td>1.0</td>
<td>[14C]ATP</td>
<td>107</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td></td>
<td>113</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td></td>
<td>109</td>
</tr>
<tr>
<td>PI-IMP</td>
<td>1.0</td>
<td>[14C]ATP</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td></td>
<td>85</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td></td>
<td>91</td>
</tr>
<tr>
<td>PI-ITP</td>
<td>1.0</td>
<td>[14C]ATP</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td></td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td></td>
<td>72</td>
</tr>
<tr>
<td>PI-ATP</td>
<td>1.0</td>
<td>[14C]ATP</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td></td>
<td>51</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td></td>
<td>82</td>
</tr>
<tr>
<td>PI-ATP</td>
<td>1.0</td>
<td>[14C]CTP</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td></td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td></td>
<td>79</td>
</tr>
</tbody>
</table>

isolated nuclei. On the other hand PI-ITP and PI-ATP were effective inhibitors of RNA synthesis in isolated nuclei with either [14C]ATP or [14C]CTP as the labeled nucleotide. Preincubation of the isolated nuclei with INOX for 2 hr prior to the polymerase assay had no effect on the RNA synthesis.

RNA Synthesis in Nuclei from Control and INOX-treated Cells. Ehrlich tumor cells were incubated in culture in the presence and absence of INOX and PI-IMP for 1.5 hr. The nuclei were then isolated from the control and treated tumor cells and used for the RNA polymerase assay. Results of these experiments are shown in Chart 4. As can be seen, RNA synthesis in the nuclei from the INOX-treated cells was markedly reduced. The nuclei from the PI-IMP-treated cells also had a marked decrease in RNA synthesis. At higher concentrations INOX was more inhibitory than was PI-IMP.

Ehrlich tumor cells were incubated in the presence and absence of INOX (1 mM). The nuclei were prepared from the tumor cells after various periods of incubation in culture, and the level of RNA synthesis in these nuclei was then determined. Results of this experiment are shown in Chart 5. There was a time dependence for the observed decrease in the level of RNA synthesis in the nuclei from INOX-treated tumor cells. The inhibition of the polymerase activity appeared to be that of classical noncompetitive inhibition, which would be consistent with simple inactivation of the polymerases. There was no change in the apparent $K_m$ for the nucleoside triphosphates with a decrease in $V_{max}$. $K_m$ for the ribonucleoside triphosphates with the RNA polymerase in the nuclei from untreated tumor cells was 0.24 mM, whereas $K_m$ for the nucleoside triphosphates with the polymerase from the INOX-treated nuclei was 0.23 mM; the $V_{max}$ was decreased. $K_m$ and $V_{max}$ parameters were determined from Eadie-Hofstee plots of the data (graphs not shown).

The time courses for RNA synthesis in the nuclei from the control and INOX-treated cells is shown in Chart 6. As can be seen, there was a larger falloff in the rate of RNA synthesis in the nuclei from the INOX-treated cells. The insert in Chart 6 shows the percentage of activity as a function of the reaction time relative to the control. Chart 7 shows the data for the reaction time course in isolated nuclei in the presence of PI-ITP; the insert in Chart 7 shows the percentage of activity as a function of the reaction time. There is a great similarity between the effects on RNA synthesis seen in the nuclei from INOX-treated cells and those seen in the nuclei treated with PI-ITP. It appears that inactivation increased during the course of reaction.

Reversibility of Inhibition of RNA in Nuclei. Tumor cells were incubated in the presence and absence of INOX (1 mM) for periods of 1 and 2 hr. In addition, tumor cells were incubated for 1 hr in the presence of INOX and then in the culture medium replaced with fresh medium without inhibitor. The nuclei were isolated from these various groups of cells, and the level of RNA synthesis was determined. These data are shown in Table 2. It did not appear that the inhibition of the RNA synthesis in the nuclei was reversible.

Chart 4. Effect of INOX and PI-IMP concentration on RNA synthesis. Ehrlich tumor cells were incubated in culture in the presence of INOX for 1.5 hr at the concentrations indicated. The nuclei were isolated, and RNA synthesis was determined as described in Chart 4. [H]UTP was the labeled substrate used in these assays.

Chart 5. Effect of incubation of Ehrlich tumor cells with INOX on RNA synthesis. Ehrlich tumor cells were incubated in culture in the presence of INOX for 1.5 hr at the concentrations indicated. The nuclei were isolated, and RNA synthesis in the nuclei was determined as described in "Materials and Methods." [H]UTP was the labeled substrate used in these assays.
polymerase activity was then determined under conditions in which either exogenous α-amanitin or actinomycin D was added to the assay mixture. The effect of α-amanitin on the residual RNA polymerase activity in the nuclei from the control and INOX-treated cells is shown in Chart 8. At concentrations of α-amanitin corresponding to the inhibition of polymerases II and III (1 × 10⁻³ μg/ml and 1000 μg/ml, respectively), slight differences were observed in the degree of inhibition between the control and INOX-treated cells. This could be due to the rates of synthesis of these species of RNA. Chart 9 shows the effect of actinomycin D on the RNA polymerase activity in the nuclei from control and INOX-treated cells. Little or no differences were observed in the degree of inhibition between the control and INOX-treated cells at the concentrations of actinomycin D tested.

**RNA Polymerase Activity in Intact Nuclei and Nuclear Extracts from INOX-treated Cells.** Ehrlich tumor cells were incubated in culture in the presence and absence of INOX. From 1 group of control and INOX-treated cells, the nuclei were prepared (17), and the RNA polymerase activity was measured. From an identically prepared group of control and INOX-treated cells, nuclear extracts were prepared (16), and the RNA polymerase activity in these extracts was then determined. Results of this experiment are shown in Table 3. There was inhibition of the RNA synthesis in the intact nuclei from the INOX-treated cells, but there was much less inhibition of the RNA polymerase activity in the nuclear extracts prepared from the INOX-treated cells. Under the conditions used to assay for RNA polymerase activity, no exogenous DNA was added to the isolated nuclei, whereas calf thymus DNA was added to the nuclear extract assay mixture.

The addition of INOX (1.6 μm) to the nuclear extract had no effect on the RNA polymerase activity. On the other hand, the addition of PI-ITP (1.0 mM) to the nuclear extract caused marked inhibition (99%) of the polymerase activity.

### Table 2

**Reversibility of inhibition of RNA polymerase activity in nuclei from INOX-treated cells**

The Ehrlich tumor cells were incubated in culture in the presence and absence of INOX (1 μm) for the times indicated. The conditions for the following groups were: Group A, cells incubated for 1 hr at 37°C; Group B, cells incubated for 1 hr at 37°C in the presence of INOX; Group C, cells incubated for 2 hr; Group D, cells incubated for 2 hr in the presence of INOX; Group E, cells incubated for 1 hr in the presence of INOX, to which fresh culture medium without INOX was then added and the incubation was continued for a second hr. The nuclei were isolated from the cells of these various groups, and the RNA polymerase activity was determined. The assays were set up in triplicate. [3H]UTP was the labeled substrate used in these assays.

<table>
<thead>
<tr>
<th>Group</th>
<th>Conditions</th>
<th>cpm/mg DNA</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Control, incubated 1 hr</td>
<td>233.5</td>
<td>100</td>
</tr>
<tr>
<td>B</td>
<td>INOX, incubated 1 hr</td>
<td>53.0</td>
<td>23</td>
</tr>
<tr>
<td>C</td>
<td>Control, incubated 2 hr</td>
<td>213.4</td>
<td>91</td>
</tr>
<tr>
<td>D</td>
<td>INOX, incubated 2 hr</td>
<td>21.8</td>
<td>9</td>
</tr>
<tr>
<td>E</td>
<td>INOX, incubated 1 hr, with fresh culture medium added for the second hr</td>
<td>53.6</td>
<td>23</td>
</tr>
</tbody>
</table>
Effect of Actinomycin D and Poly(dA-dT) on RNA Synthesis in Nuclei from Control and INOX-treated Cells. Since the nuclear extracts from the INOX-treated cells showed little or no inhibition of the RNA polymerase activities, the possibilities existed that the inhibition of the RNA polymerase was reversed by simple dilution of the inhibitor or that the inhibition was reversed because, in the assay of the nuclear extracts, exogenous DNA template was added. For testing the latter possibility, experiments were carried out in a manner similar to those of Herzog and Farber (12). RNA synthesis was determined in nuclei from control and INOX-treated cells. Actinomycin D (20 μg/ml) was added to the nuclei to inhibit RNA synthesis on the endogenous template. Poly(dA-dT), which would not bind actinomycin D, was added as the exogenous template. The results of these experiments are shown in Table 4. With endogenous DNA as template, RNA synthesis in the nuclei from INOX-treated cells was inhibited 55%. However, when actinomycin D and...
poly(dA-dT) were added, the level of RNA synthesis with exogenous poly(dA-dT) as template was 96% of the control value. These results clearly show that the effect of INOX on RNA synthesis was not due to the inhibition of the RNA polymerase enzymes.

Incorporation of \([^{14}C]INOX\) into Nuclei of Ehrlich Tumor Cells. Ehrlich tumor cells were incubated in culture in the presence of 2 concentrations of \([^{14}C]INOX\) (1.2 x 10^6 cpm and 2.4 x 10^6 cpm; 550 mCi/mmol) for 1.5 hr. The nuclei were isolated. Radioactivity in the nuclei and the cytosolic fraction was determined. At the lower \([^{14}C]INOX\) concentration, 3.5% of the INOX was taken up by the tumor cells, whereas 3.8% of the INOX was taken up at the higher concentration. Of the amount of \([^{14}C]INOX\) transported into the tumor cells, 14 and 17% of the \([^{14}C]INOX\) were found in the nuclear fraction at the 2 concentrations.

DISCUSSION

Previous studies had shown that INOX inhibited RNA and DNA synthesis in Ehrlich tumor cells in culture (7). The inhibition of DNA synthesis was correlated with the inhibition of ribonucleotide reductase (5). In the present study we have investigated the nature of the inhibition of RNA synthesis by INOX. The inhibition of RNA synthesis was not the result of selective inhibition of 1 of the RNA polymerases (I, II, or III). There was inhibition of the synthesis of precursor ribosomal RNA as well as of the other high-molecular-weight RNA's synthesized.

The addition of INOX or PI-IMP to isolated nuclei did not result in inhibition of RNA polymerase activity, which agreed with the data reported by Cysyk and Adamson (9). In addition, INOX did not inhibit the polymerase activity in the nuclear extracts. However, PI-ATP and PI-ITP were effective inhibitors of RNA synthesis when they were added to the isolated nuclei. PI-ITP and PI-IMP were effective inhibitors of DNA synthesis in the nuclear extracts. The fact that INOX or PI-IMP did not inhibit the RNA synthesis in isolated nuclei or nuclear extracts but did reduce RNA synthesis in the nuclei from treated cells suggested that INOX and PI-IMP were metabolized to a compound that was the ultimate inhibitor. With the use of \([^{14}C]INOX\), it had been previously shown that INOX was phosphorylated both in intact tumor cells and by cell-free extracts to the diphosphate and triphosphate derivatives (11). These studies also indicated that \([^{14}C]INOX\) was incorporated into the RNA of intact tumor cells. This incorporation was inhibited by actinomycin D (11). There was significant accumulation of radioactivity from \([^{14}C]INOX\) into the nuclei of the Ehrlich tumor cells (present study). The inhibition of RNA synthesis in INOX-treated cells was not reversible by resuspending the treated tumor cells in fresh culture medium in the absence of INOX. However, nuclear extracts from INOX-treated cells showed slight inhibition of the RNA polymerases, suggesting that in cell-free extracts the inhibition was reversible. The reason for the apparent reversal of the inhibition of the RNA polymerase activity in the nuclear extracts was that, in fact, the RNA polymerase enzymes were not inhibited. Rather, it appears that the incorporation of the INOX into RNA caused termination of the growing RNA chain on the template and rendered the template unavailable for further RNA synthesis. Strong evidence for this conclusion was provided by the experiment (Table 4) that showed that complete RNA polymerase activity was present in nuclei from INOX-treated cells if exogenous DNA as template [in this case the poly(dA-dT)] was added.

The results of these studies clearly show that the modes of inhibition of INOX on RNA synthesis and ribonucleotide reductase are entirely different. The inhibition of ribonucleotide reductase is the result of the inhibition of the enzyme, and this inhibition cannot be reversed by Sephadex G-25 chromatography and ultrafiltration (7). Further, INOX and PI-IMP were capable of inhibiting ribonucleotide reductase activity in cell-free extracts. On the other hand the inhibition of RNA synthesis by INOX required that INOX be metabolized to the triphosphate. INOX had no effect on RNA synthesis in isolated nuclei or nuclear extracts. Only PI-ITP inhibited the RNA synthesis in isolated nuclei or nuclear extracts. In this case the mode of inhibition of RNA synthesis by the dialdehyde derivative of inosine was not due to the interaction of the dialdehyde moieties with the amino groups on the protein (RNA polymerases) to form Schiff bases but rather was due to chain termination of the growing RNA chain or impairment of template function.

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