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# Inhibition of Ribonucleotide Reductase Activity and Nucleic Acid Synthesis in Tumor Cells by the Dialdehyde Derivatives of Inosine (NSC 118994) and Inosinic Acid[1]

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## SUMMARY

Periodate-oxidized inosine (Inox; NSC 118994) and periodate-oxidized 5′-inosinic acid (PI-IMP) were prepared and studied for their effects on ribonucleotide reductase activity in partially purified extracts from Ehrlich tumor cells and on nucleic acid synthesis in intact tumor cells in culture.

Ribonucleotide reductase activity in cell-free extracts from Ehrlich tumor cells was inhibited by Inox and PI-IMP. PI-IMP was more inhibitory to the reductase activity than was Inox. Furthermore, the inhibition of ribonucleotide reductase activity by Inox and PI-IMP was greater for cytidine-5′-diphosphate reductase activity than for adenosine-5′-diphosphate reductase activity. The ribonucleotide reductase activity in cell-free extracts prepared from Ehrlich tumor cells treated with Inox or PI-IMP in culture was decreased compared with the activity in the extracts from untreated cells.

Incorporation of labeled cytidine into the RNA and DNA of Ehrlich tumor cells in culture was inhibited by both Inox and PI-IMP. The conversion of cytidine to deoxycytidine nucleotides in the acid-soluble pool was likewise inhibited.

These data indicate that Inox andPI-IMP inhibit the ribonucleotide reductase step as one of the sites of action of these compounds. However, the inhibition of RNA synthesis indicates that there must be additional sites of action of these nucleoside analogs.

## INTRODUCTION

Previous studies from this laboratory have shown that the dialdehyde derivatives of adenosine, AMP, and ATP inhibited ribonucleotide reductase activity (4) and that the dialdehyde derivatives of adenosine and AMP inhibited nucleic acid synthesis in intact Ehrlich tumor cells (6). The dialdehyde derivatives of β-D-ribosyl-6-methylthiopurine have been shown to be both carcinostatic and immunosuppressive (1). Kimball's group reported that thymidylate kinase, DNA polymerase (13), and RNA polymerase (16) were inhibited by this compound.

Since the dialdehyde derivative of inosine (NSC 118994) had been shown to inhibit nucleic acid synthesis in L1210 cells and to inhibit tumor growth *in vivo*,[2] and this compound (NSC 118994) has recently been started in Phase I clinical trials (11), we investigated the effect of the inosine derivative on ribonucleotide reductase activity in cell-free extracts and on this step in intact tumor cells. PI-IMP[3] was included in these studies because Cohen's group (17) had reported that the 1-β-D-arabinofuranosyladenosine 5′-monophosphate was more inhibitory to L-cells than 1-β-D-arabinofuranosyladenosine.

## MATERIALS AND METHODS

**Preparation of Inox and PI-IMP.** Inox and PI-IMP were prepared by the method of Khym and Cohn (12). Briefly, 500 mg of inosine or IMP were dissolved in 150 ml of 0.01 M sodium acetate (pH 5.0) and 50 ml of 0.1 M $NaIO_4$ were added. The reaction was carried out for 2 hr at room temperature in the dark. Excess glycerol (0.2 ml) was added to discharge the unreacted periodate remaining. This reaction was carried out for 1 additional hr. The reaction mixture was put over a Dowex 1-formate column (26 x 5 cm). The Inox was eluted with 0.02 M formic acid. PI-IMP was eluted with a linear gradient of 0.02 to 4.0 M formic acid. Fractions (10 ml/fraction) were collected. All fractions having absorbances at 260 nm greater than 2 were pooled and taken to dryness on a rotary evaporator. The product was dissolved in water and again taken to dryness. This was repeated 4 times until the pH of the solution showed that the formic acid had been removed. In the preparations used in these studies, a 80% yield of Inox was obtained and a 65% yield of PI-IMP was obtained. The products were tested to show the absence of iodate or periodate by the sensitive starch-iodide test (20). The presence of the aldehyde functional groups was shown by the method of Zamecnik, *et al.* (21). Paper chromatography in butanol-$H_2O$ showed the characteristic streaking of the dialdehyde compound (12). High-pressure liquid chromatography (HPLC) was further used to characterize the products. The preparations of Inox and PI-

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[3] The abbreviations used are: PI-IMP, periodate-oxidized derivative of 5′-inosinic acid; Inox, periodate-oxidized derivative of inosine (NSC 118994).

IMP were subjected to high-pressure liquid chromatography on a Whatman Partisil SAX column that had been equilibrated with 0.1 M boric acid, pH 5.0. The flow rate was 2 ml/min. Both Inox and PI-IMP eluted at 1.8 min (essentially, the void volume). Inosine eluted at 3.8 min while IMP was not eluted from the column until the ionic strength of the eluting buffer was increased with the addition of 0.5 M ammonium phosphate (pH 4.8) from the 2nd pump of the gradient system. IMP eluted at 14.0 min. By analysis on the high-pressure liquid chromatograph, the presence of inosine or IMP was not detected. Hypoxanthine was not detected by paper chromatography in either the Inox or PI-IMP preparations.

**Assay of Ribonucleotide Reductase Activity.** The ammonium sulfate fraction prepared from Ehrlich tumor cells was used as the source of ribonucleotide reductase (4). The CDP reductase activity was determined by the method of Steeper and Steuart (19), and the ADP reductase activity was determined by the method of Cory *et al.* (7). All enzyme assays were run in triplicate.

**Effect of Inox and PI-IMP on Nucleic Acid Synthesis in Ehrlich Tumor Cells in Culture.** The Ehrlich tumor cells were taken under sterile conditions from mice 5 to 7 days after tumor inoculation. The culture conditions were as previously described (6). The tumor cells were incubated in the presence or absence of the inhibitors for 1.5 hr. [$^{14}$C]Cytidine (374.5 mCi/mmole; 0.2 $\mu$Ci/flask) was added to each culture flask, and the incubation was carried out for an additional 30 min. The cells were collected by centrifugation and subjected to the Schmidt-Thannhauser procedure (18) to separate the acid-soluble, RNA, and DNA fractions. For measurement of the conversion of cytidine nucleotides to deoxycytidine nucleotides, the acid-soluble fraction was treated with snake venom to hydrolyze the cytidine and deoxycytidine nucleotides to cytidine and deoxycytidine which were then separated on Dowex 1-borate columns (6). The level of conversion of [$^{14}$C]cytidine to CMP, CDP, and CTP in the acid-soluble fraction was determined by chromatography on Whatman No. 3MM filter paper (6).

**Ribonucleotide Reductase Activity in Cell-free Extracts from Inox and PI-IMP-treated Cells.** Ehrlich tumor cells were incubated for 2 hr in culture in the presence and absence of either Inox (1.0 mM), PI-IMP (2.8 mM), or hydroxyurea (3.0 mM). The cells were collected by centrifugation and homogenized in 0.02 M Tris-HCl (pH 7.0) containing 1 mM dithioerythritol. The homogenate was centrifuged at 27,000 × *g* for 1 hr, and the CDP reductase activity in the cell-free extracts was assayed as described earlier.

In other experiments, the cell-free extracts were put over Sephadex G-25 columns (27 x 1 cm), and the protein eluted with 0.02 M Tris-HCl (pH 7.0) containing 1 mM dithioerythritol. The protein fractions eluted with the void volume were then concentrated by ultrafiltration on an Amico PM-10 membrane.

**Miscellaneous.** Thymidine kinase activity was determined by the method of Breitman (2). Deoxycytidylate deaminase activity was determined by the method of Maley and Maley (14) using [$^{3}$H]dCMP as substrate. Protein, RNA, and DNA determinations were made by the methods of Lowry *et al.* (13), Hurlbert *et al.* (10), and Burton (3), respectively.

## RESULTS

**Inhibition of Ribonucleotide Reductase Activity by PI-IMP and Inox.** Inox and PI-IMP were tested as inhibitors of CDP reductase (Chart 1) and ADP reductase (Chart 2) activity. With both compounds, CDP reductase activity was inhibited to a greater extent than was ADP reductase activity. Furthermore, PI-IMP was more inhibitory than was Inox to the ribonucleotide reductase activity in partially purified cell-free extracts.

In an attempt to determine the nature of the inhibition of the reductase by Inox and PI-IMP, data were collected for analysis by Dixon plots (8) to determine whether the inhibition was of the competitive or noncompetitive type. Analysis of the data (Chart 3, *A* and *B*) indicated that the inhibition did not fall strictly into either the competitive or noncompetitive type, since the lines did not intersect either above the axis or on the axis for competitive and noncompetitive inhibitors, respectively. Further analysis showed that the inhibition by these compounds was not reversible. Partially purified ribonucleotide reductase was incubated with 2.84 mM Inox and 0.8 mM PI-IMP for 30 min at 0°. The samples,

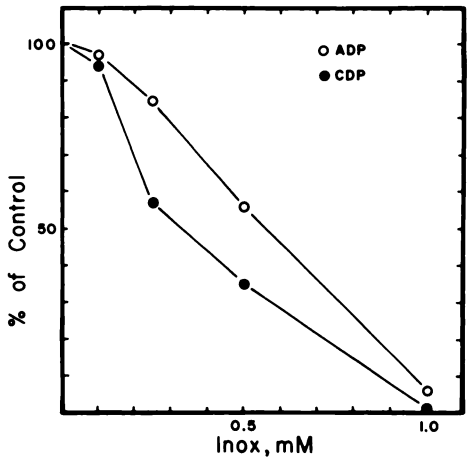


Chart 1. Inhibition of CDP reductase and ADP reductase activity by Inox. The CDP reductase and ADP reductase activities were assayed as described in "Materials and Methods," except that Inox was added at the concentrations indicated. The 20 to 40% ammonium sulfate fraction was the enzyme fraction used. All assays were run in triplicate.

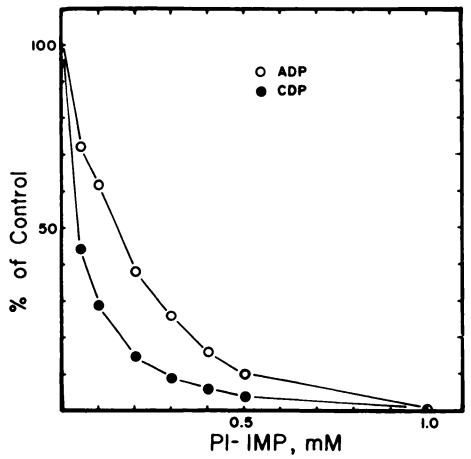


Chart 2. Inhibition of CDP reductase and ADP reductase activities by PI-IMP. The experiment was set up as described for Chart 1 except that PI-IMP was used as the inhibitor.

including a control, were put over Sephadex G-25 columns (27 x 1 cm), and the protein eluted with 0.02 M Tris-HCl (pH 7.0). Fractions (1 ml) were collected, and the fractions eluting with the void volume were pooled and concentrated on a Amicon PM-10 membrane. The concentrated protein peaks were used for the reductase assays shown in Table 1. It was observed that the samples treated with Inox or PI-IMP had lower reductase activities than did the control sample. The irreversible nature of the inhibition even after Sephadex G-25 chromatography and Amicon PM-10 ultrafiltration would explain why the Dixon plots (8) do not demonstrate strictly either competitive or noncompetitive inhibition.

**Ribonucleotide Reductase Levels in Inox and PI-IMP-treated Tumor Cells.** Ehrlich tumor cells were incubated in culture medium with and without the addition of Inox and PI-IMP. After a 2-hr incubation, the tumor cells were collected by centrifugation and cell-free extracts were prepared from the control and treated cells. The CDP reductase activity was measured in these extracts. The results of this experiments are shown in Table 2. It was observed that the cell-free extracts prepared from the Inox- and PI-IMP-treated cells had much lower levels of ribonucleotide reductase activity. As a control, tumor cells were treated with hydroxyurea (2 mM), and cell-free extracts were prepared in a similar manner. Under these conditions, there was no decrease in reductase activity in the hydroxyurea-treated cells. This concentration of hydroxyurea inhibited thymidine incorporation into DNA by more than 90% (data not shown).

To demonstrate further the effect of these compounds on the reductase activity in intact cells, cell-free extracts were prepared from control, Inox-treated, and PI-IMP-treated cells as above. In this case, the cell-free extracts were put over Sephadex G-25 columns (27 x 1 cm) and eluted with 0.02 M Tris-HCl (pH 7.0) containing 1 mM dithioerythritol. The protein peaks eluting in the void volume were concentrated on an Amicon PM-10 membrane (Table 3). These crude extracts prepared from the Inox- and PI-IMP-treated tumor cells that were subjected to gel chromatography and ultrafiltration showed a marked decrease in the CDP reductase and ADP reductase activities.

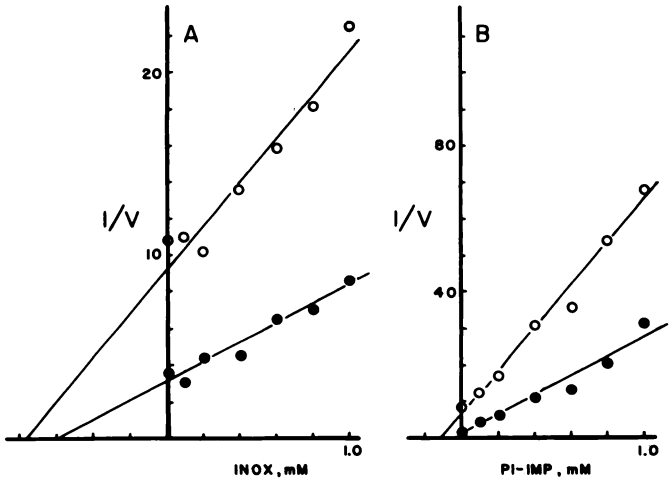


Chart 3. Dixon plots of Inox and PI-IMP effects on reductase activity. The compounds, Inox and PI-IMP, were added to the standard reaction mixtures for the CDP reductase, except that the final substrate concentrations were 2.5 μM (○) and 12.5 μM (●), respectively. All assays were carried out in triplicate. The lines drawn through the data points were determined by a least-squares analysis of the data.

Table 1

*Effect of Inox and PI-IMP on partially purified reductase*

The ammonium sulfate fraction (20 to 40%), containing the reductase activity, was incubated with Inox (2.84 mM) and PI-IMP (0.8 mM) for 30 min on ice. The samples were then put over a Sephadex G-25 column (27 x 1 cm) and eluted with 0.02 M Tris-HCl, pH 7.0. The protein peaks eluting in the void volume were concentrated by ultrafiltration on Amicon PM-10 membranes. The control sample was carried through Sephadex G-25 chromatography and ultrafiltration steps. The reductase assays were run in triplicate as described in the "Materials and Methods."

| | nmoles of product/30 min/mg protein | |
|---|---|---|
| | CDP | ADP |
| Control | 0.75 (100)[a] | 0.47 (100) |
| Inox | 0.35 (46) | 0.20 (42) |
| PI-IMP | 0.43 (57) | 0.18 (39) |

[a] Numbers in parentheses, percentage of control values.

Table 2

*Ribonucleotide reductase activity in PI-IMP- and Inox-treated cells*

Each group of culture flasks was set up in quadruplicate as previously described (6). The final concentrations of PI-IMP, Inox, and hydroxyurea were 2.8, 1.0, and 3.0 mM, respectively. At the end of the incubation time, the cells from 2 control flasks were pooled as were the cells from 2 experimental flasks. Duplicate cell-free extracts were prepared. The enzyme activity was determined as described in "Materials and Methods." All reductase assays were set up in triplicate.

| Compound | CDP reductase activity (nmoles/30 min/mg protein) | % of control |
|---|---|---|
| None | 0.156 | 100 |
| PI-IMP | 0.017 | 11 |
| Inox | 0.025 | 16 |
| Hydroxyurea | 0.157 | 100 |

Table 3

*Effect of Inox and PI-IMP on reductase activity in intact tumor cells*

The flasks were set up as described in Table 2. In these experiments, the final concentrations of Inox and PI-IMP were 1.5 and 3.2 mM, respectively. The cell-free extracts were put over Sephadex G-25 columns (27 × 1 cm) and eluted with 0.02 M Tris-HCl (pH 7.0) containing 1 mM dithioerythritol. The protein in the void volume was concentrated by ultrafiltration on Amicon PM-10 membranes. Reductase assays were run in triplicate.

| | nmoles/30 min/mg protein | |
|---|---|---|
| | CDP | ADP |
| Control | 0.113 (100)[a] | 0.093 (100) |
| Inox | 0.015 (13) | 0.029 (38) |
| PI-IMP | 0.052 (46) | 0.034 (45) |

[a] Numbers in parentheses, percentage of control values.

**Inhibition of Nucleic Acid Synthesis in Ehrlich Tumor Cells by Inox and PI-IMP.** Ehrlich tumor cells were incubated in culture with Inox and PI-IMP, and the effect of these compounds on nucleic acid synthesis was studied. Chart 4 shows the effect of various concentrations of Inox on RNA and DNA synthesis. It was observed that the inhibition of RNA and DNA synthesis paralleled each other. As a measure of the ribonucleotide reductase activity in the intact cell, the conversion of [$^{14}$C]cytidine to [$^{14}$C]de-

oxycytidine nucleotides was measured in the acid-soluble fraction. These data are also included in Chart 4, and it is seen that the decrease in the formation of deoxycytidine paralleled the decrease in nucleic acid synthesis.

Chart 5 shows the effect of the various concentrations of PI-IMP on RNA and DNA synthesis in the Ehrlich tumor cells. In this case, the inhibition of DNA synthesis at the higher concentrations of PI-IMP was greater than the inhibition of RNA. The inhibition of deoxycytidine formation paralleled the inhibition of DNA synthesis.

The inhibition of DNA synthesis by Inox was time dependent. Incubation of the cells with Inox (0.48 mM) for 1 and 2 hr, followed by a 30-min labeling period, gave inhibition of DNA synthesis of 37 and 63%, respectively. On the other hand, inhibition of DNA synthesis by PI-IMP did not appear to increase with time. The percentages of inhibition of DNA synthesis by PI-IMP for the 1- and 2-hr incubation periods were 63 and 66%, respectively.

Inox and PI-IMP had no effect on the conversion of [$^{14}$C]cytidine to [$^{14}$C]CTP in the tumor cells (Table 4). [$^{14}$C]Cytidine was not detected in the acid-soluble fraction prepared from the Ehrlich tumor cells.

**Other Possible Sites of Action.** Thymidine kinase activity was not inhibited by either Inox or PI-IMP. This lack of inhibition was observed in both cell-free extracts and intact cells by the measurement of [$^{14}$C]thymidine conversion to TMP, TDP, and TTP. The data from the intact cells also indicated that TMP and TDP kinases were not inhibited by these compounds.

Furthermore, the level of inhibition of incorporation of [$^{14}$C]deoxyuridine and [$^{14}$C]thymidine in DNA of the tumor cells was the same, indicating that thymidylate synthetase was not an intracellular site of action.

Deoxycytidylate deaminase activity was not inhibited by these compounds as determined in crude cell-free extracts prepared from the Ehrlich tumor cells.

Neither hypoxanthine-guanine phosphoribosyltransferase nor adenine phosphoribosyltransferase were inhibited by Inox or PI-IMP in intact Ehrlich tumor cells. This was determined by measuring the uptake and conversion of [$^{14}$C]guanine and [$^{14}$C]adenine to the corresponding nucleotides. In each case, more than 90% of the labeled purine in the acid-soluble fraction was converted to the nucleotide derivative. Less than 5% of the [$^{14}$C]adenine added to the culture medium was taken up by the Ehrlich tumor cells. Approximately 0.7% of the [$^{14}$C]guanine added to the culture flasks was taken up by the tumor cells. The presence of Inox or PI-IMP in the culture medium had no effect on this conversion, although the incorporation of [$^{14}$C]adenine and [$^{14}$C]guanine into RNA and DNA was inhibited in these experiments.

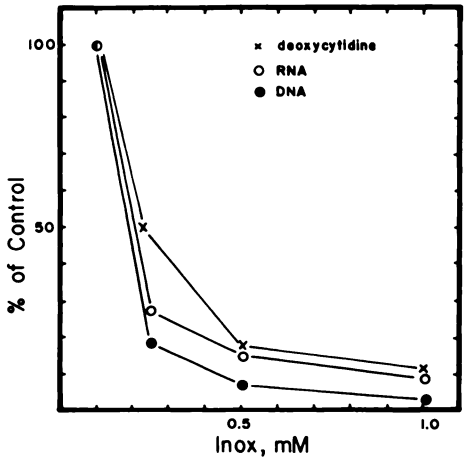


Chart 4. Inhibition of nucleic acid synthesis in Ehrlich tumor cells by Inox. Ehrlich tumor cells were incubated in culture in the presence and absence of Inox for 1.5 hr at 37°. [$^{14}$C]Cytidine (274.5 mCi/mmole; 0.2 μCi/flask) was added and the incubation was carried out for an additional 30 min. The tumor cells were subjected to the Schmidt-Thannhauser procedure (18) to separate the acid-soluble, RNA and DNA fractions. The conversion of cytidine to deoxycytidine nucleotides (×) in the acid-soluble fraction was determined as previously described (6). No inhibition is seen at ◐.

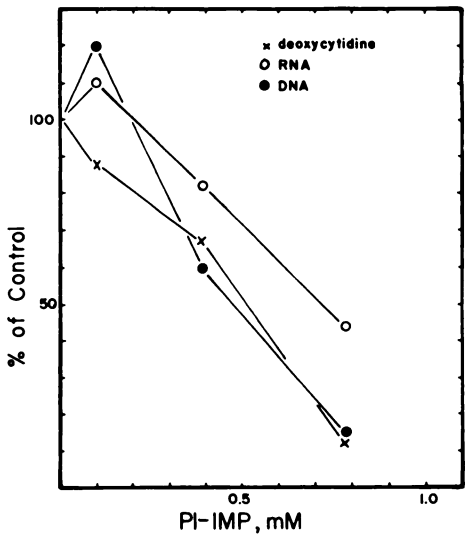


Chart 5. Inhibition of nucleic acid synthesis in Ehrlich tumor cells by PI-IMP. Ehrlich tumor cells were incubated in culture in the presence and absence of PI-IMP. The cells were treated as described in Chart 3.

Table 4

*Distribution of cytidine nucleotides in acid-soluble fraction*

The acid-soluble fractions from the Inox- and PI-IMP-treated cells and from control cells were extracted with ether to remove the trichloroacetic acid. The samples were lyophilized, dissolved in 0.2 ml $H_2O$, and chromatographed on Whatman No. 3MM filter paper as previously described (6).

| | % distribution | | |
|---|---|---|---|
| Sample | CMP | CDP | CTP |
| Control | 12 | 21 | 67 |
| Inox | 12 | 13 | 75 |
| PI-IMP | 13 | 17 | 70 |

## DISCUSSION

A study of the effects of Inox was undertaken because this compound was shown to inhibit nucleic acid synthesis in L1210 cells[2] and, more importantly, because this compound has been started on Phase I clinical testing (11). An understanding of the specific site(s) of action of this compound could aid in the use of this compound in the treatment of certain tumors and especially in the design of combination chemotherapy protocols.

The results of these studies indicate that the ribonucleotide reductase step is one of the major sites of inhibition by Inox and PI-IMP. This was shown by the effects of these compounds on CDP reductase and ADP reductase activities in partially purified enzyme fractions (Charts 1 and 2). The inhibition of ribonucleotide reductase activity in partially purified fractions by Inox and PI-IMP was at least partially irreversible since the full activity could not be recovered after Sephadex G-25 chromatography and ultrafiltration to remove the periodate-oxidized compounds (Table 1).

In addition, cell-free extracts prepared from Inox- and PI-IMP-treated tumor cells had a marked decrease in ribonucleotide reductase activity (Table 2). The control experiment with hydroxyurea (a known reversible inhibitor of ribonucleotide reductase) indicated that these compounds were probably acting in an irreversible manner. Furthermore, the cell-free extracts prepared from the Inox- and PI-IMP-treated cells and subjected to Sephadex G-25 chromatography and ultrafiltration still showed a marked decrease in the ribonucleotide reductase activity (Table 3). These data indicate rather strongly that these compounds have an irreversible effect on ribonucleotide reductase.

That the inhibition of DNA synthesis by these periodate-oxidized compounds was due to the inhibition of ribonucleotide reductase was further supported by the analysis of the acid-soluble fractions of the control and drug-treated cells. Both Inox and PI-IMP caused a decrease in the conversion of cytidine to deoxycytidine which, of course, is a measure of intracellular ribonucleotide reductase activity (Charts 5 and 6).

We have recently reported that there was an excellent correlation between the inhibition of ribonucleotide reductase activity in the intact cells as measured by this method and the inhibition of DNA synthesis by the periodate-oxidized derivatives of adenosine, 6-*N*-methyladenosine, purine riboside, and inosine. This strongly implicated the reductase step as a common site of action by these compounds (5). In addition, these data showed that complete inhibition of DNA synthesis was obtained when the conversion of cytidine to deoxycytidine was inhibited only 70%. That is, complete inhibition of ribonucleotide reductase is not essential for complete inhibition of DNA synthesis.

Gutensohn and Huber (9) have reported that PI-IMP inhibited the hypoxanthine-guanine phosphoribosyl transferase activity in cell-free extracts prepared from brain and RBC hemolysates. However, the experiments carried out with [$^{14}$C]guanine ruled out hypoxanthine-guanine phosphoribosyl transferase as a site of inhibition of the periodate-oxidized compounds in intact cells.

Other sites of action have been ruled out. Thymidine kinase and deoxycytidylate deaminase activities were not inhibited by either Inox or PI-IMP by direct assay. Indirectly, in the intact cells the TMP and TDP kinases and thymidylate synthetase were also determined not to be inhibited by these compounds. It has not yet been determined where the site of inhibition of RNA synthesis occurs. This aspect and the study of the metabolism of these dialdehyde derivatives are currently under study.

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