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METABOLISM OF THE DIALDEHYDE DERIVATIVE OF INOSINE (NSC-118994): TRANSPORT, DISTRIBUTION AND INCORPORATION INTO RNA OF EHRLICH ASCITES TUMOR CELLS*

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Abstract—Inox (NSC-118994) was studied with the use of the ^{14}C-labeled compound to further delineate its intracellular metabolism and particularly its effect on DNA and RNA synthesis. Its transport was shown not to be competitively inhibited by the major ribonucleosides or deoxynucleosides. In addition, Inox was shown to be transported into the cell without degradation. Ouabain did not affect its transport. Inox was found to be a component of the nucleoside and nucleotide pools of the tumor cell. It is acted upon by cellular kinases to produce the nucleotide derivative. Significant incorporation occurred in the RNA fraction of the tumor cells. Actinomycin D inhibited the incorporation of [^{14}C]Inox into RNA. This incorporation was found to occur primarily in small molecular weight RNA species.

Inox-treated tumor cells were shown to have a quantitative decrease in ribosomal precursor RNA. There appeared to be no incorporation of Inox into the DNA of the Ehrlich tumor cells.

Inox was shown to be an effective antitumor agent *in vivo* with a 70% reduction of Ehrlich ascites tumor cells in 2 days.

A series of carbohydrate and nucleoside periodate-oxidized derivatives were prepared and several of these were shown to have significant antitumor effects in experimental mouse systems [1]. Kimball *et al.* [2] showed that the periodate-oxidized derivative of β-D-ribosyl-6-methylthiopurine (6-MeMPR-OP) inhibited thymidylate kinase and DNA polymerase from Ehrlich tumor cells and more than doubled the life span of tumor-bearing mice. Bell *et al.* [3] further showed that 6-MeMPR-OP had antitumor and immunosuppressive activity. These studies were followed by reports showing that 6-MeMPR-OP inhibited purified preparations of DNA-dependent RNA polymerase [4, 5] and ribonuclease [6] through the formation of Schiff-base intermediates with the ϵ-NH_2 group of lysyl residues at or near the active sites of these enzymes. Bell and Gisler [7] subsequently showed, using ^{35}S-labeled MeMPR-OP, that this drug was rapidly excreted by mice but was also found to be bound in several tissues.

We have reported that the periodate-oxidized derivatives of nucleosides inhibit nucleic acid synthesis in Ehrlich ascites tumor cells [8–10]. They were shown to inhibit both DNA and RNA synthesis. Ribonucleotide reductase activity was shown to be inhibited by these dialdehyde compounds [9–11]. The inhibition of DNA synthesis correlated with the inhibition of ribonucleotide reductase activity [9] and indicated that this was one of the sites of action responsible for the inhibition of DNA synthesis.

Phase I clinical trials have been started on Inox (NSC-118994). The initial results were encouraging enough to warrant recommendation of the use of this compound in Phase II trials [12]. Pharmacological studies in mice, rats and monkeys had indicated that much of the Inox was rapidly excreted intact via the urinary route [13]. The use of [^{14}C]Inox showed that the majority of the [^{14}C]Inox in the plasma was trichloroacetic acid-precipitable indicating that the [^{14}C]Inox was protein-bound presumably through Schiff-base intermediates.

In vitro studies showed that Inox, through its two aldehyde moieties, could cross-link protein molecules [14]. Other studies showed that the cytotoxicity of Inox was cell-cycle dependent being most active during late G_1-early S phase [15].

From the studies carried out to date on the dialdehyde derivatives of nucleosides, several interesting differences have been observed between compounds which cannot be related *only* to the dialdehyde moiety. The study from Southern Research Institute showed that Inox and 6-MeMPR-OP had quantitatively different effects on L1210 cells.‡ Further, this study showed that H.Ep-2 cells were not as sensitive to Inox as were the L1210 cells. However, 6-MeMPR-OP was inhibitory to both L1210 and H.Ep-2 cells. Our studies have shown that nucleic acid synthesis in Ehrlich tumor cells was inhibited by the dialdehyde derivatives of adenosine and inosine but was much less sensitive to the dialdehyde derivatives of purine riboside and guanosine. In addition, our studies have indicated quantitative differences in the inhibition of RNA and DNA synthesis

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by the dialdehyde derivatives of inosine and IMP and adenosine and AMP.

Rational clinical use of this compound, as well as possible future use in combination with other antitumor drugs necessitate further information about the uptake, metabolism and sites of action of this compound. The purpose of this report is to elaborate on the cellular uptake, metabolism and utilization of Inox with particular emphasis on its incorporation into RNA.

MATERIALS AND METHODS

Preparation of [^{14}C]Inox. [U-^{14}C]Inosine (550 mCi/m-mole) was purchased from Amersham Searle. The periodate oxidation of [^{14}C]inosine was carried out by the method of Khym & Cohn [16]. The reaction mixture consisted of 50 μCi inosine in 0.1 M sodium meta periodate and 0.1 M sodium acetate buffer, pH 5.0. This reaction was carried out at room temperature for 1 hr in the dark. Ethylene glycol (0.02 ml) was then added to discharge the unreacted periodate. This reaction was carried out at room temperature for 30 min. The Inox was purified by high pressure liquid chromatography (HPLC). A Whatman Partisil column SAX 10/25, which had been equilibrated with 0.1 M sodium borate, pH 5.0 was used. The sample was eluted isocratically with 0.1 M sodium borate, pH 5.0 with a flow rate of 2 ml/min. Fractions (1 ml) were collected. Retention time for the standard compounds were as follows: Inox, 1.8 min; Inosine, 5.6 min; Iodate, retained on the column. The [^{14}C]Inox eluted from the HPLC was checked for purity on PEI-thin layer cellulose with H_2O as a solvent. The Rf's for standards were Inox, <0.02; Hypoxanthine, 0.43; and Inosine, 0.56. The purified [^{14}C]Inox was found to be >99% Inox.

Ehrlich tumor cells in culture. The Ehrlich tumor cells were grown in female mice (ICR) by weekly inoculation of recipient mice with 0.2 cc of tumor cells as taken from mice. The mice were purchased from Lab Supply Company, Indianapolis, IN.

All cell culture experiments were done under sterile conditions as previously described [17]. The cells were harvested by centrifugation and processed by the Schmidt-Thannhauser procedure for acid-soluble, RNA and DNA fractions [18]. When measuring [^{14}C]Inox incorporation, the cultures contained 0.025 μCi/ml (45 pmoles/ml) [^{14}C]Inox in the culture medium.

Preparation of cell-free extract and assay of Inox phosphorylation. For the preparation of the crude cell-free extract, Ehrlich tumor cells were taken, washed with 0.15% NaCl, and homogenized in 0.02 M Tris HCl, pH 7.0 (3 vol. buffer/vol. packed cells) with a motor driven teflon pestle. The homogenate was centrifuged for 1 hr at 30,000 *g*. The supernatant fluid (5 ml) was dialyzed against 0.02 M Tris–HCl, pH 7.0 for 2 hr. These procedures were all carried out with ice-cold buffer and/or 4°.

The assay mixture in a final vol. of 0.2 ml contained: [^{14}C]Inox (150,000 cpm); ATP (1 mM); Tris–HCl buffer, 0.02 M, pH 7.0 and crude extract (100 μl, 7.5 mg/ml). The reaction was carried out for 1 hr at 37°. The reaction was stopped by the addition of 5% trichloroacetic acid (0.5 ml). Controls were run in which the addition of the trichloroacetic acid preceded the addition of [^{14}C]Inox to the reaction mixture. The acid-insoluble pellets were removed by centrifugation, and the pellets washed with 0.5 ml of 5% trichloroacetic acid. The acid washings were added to the previous supernatant fluids. The samples were neutralized by ether extraction of the trichloroacetic acid. The neutralized samples were put over Dowex-1-formate columns (Pasteur pipettes) and eluted as follows: H_2O (5 ml); 2 M formic acid (3 ml); and 3 M HCl (3 ml). Aliquots were taken for radioactivity measurements and corrections made for the quench caused by the 3 M HCl. All assays were carried out in triplicate.

Chromatography of acid-soluble pools. (a) Dowex-1-Formate: The Ehrlich tumor cell pellets were extracted with one ml cold 5% trichloroacetic acid (TCA). This was repeated two additional times. The acid-soluble fractions were extracted with two vol. of ether (four times) to remove the TCA. The samples were lyophilized to dryness, resuspended in 0.5 ml of H_2O and placed over a Dowex-1-formate column (pasteur pipette, 1.7 × 0.5 cm) and eluted as follows: 5 ml. H_2O; 3 ml, 2 M formic acid; and 3 ml, 3 M HCl. Standards eluted from the column were H_2O eluant—Inox, inosine, adenosine and guanosine; formic acid eluant—PI-IMP, IMP, AMP, GMP; HCl eluant—ADP, ATP, GDP, GTP, PI-ITP.

(b) High Pressure Liquid Chromatography: Acid-soluble fractions from the tumor cells were separated by high pressure liquid chromatography. The eluting buffers in the solvent reservoirs were 0.1 M sodium borate, pH 5.0 and 0.5 M potassium phosphate, pH 4.82. The low ionic strength buffer was run for 10 min after injection of sample and then the linear gradient was begun. This allowed for improved separation of Inox from nucleotides. The linear gradient was developed with a 20 min program. Fractions (1.0 ml) were collected. Standard compounds had the following retention times: Inox, 1.8 min; hypoxanthine, 1.8 min; inosine, 5.6 min; PI-IMP, 1.8 min; purine 5'monophosphates ~ 18 min; purine 5'-diphosphates ~ 27 min, purine 5'-triphosphates ~ 34 min. Acid-soluble extracts were treated as previously described except that the lyophilized samples were resuspended in 0.2 ml H_2O and 50 μl sample was injected onto the column.

Isolation and fractionation of RNA. Cells were harvested either directly from the tumor-bearing mice or after tumor cells were put into culture and the RNA was extracted by the method of Wilkinson *et al.* [19]. The RNA was fractionated into small mol. wt, 18S, 28S and precursor species on linear sucrose gradients (5–47%, 36 ml) in a Beckman L-65 preparative ultracentrifuge. All samples were centrifuged in a SW27 rotor at 26,000 rpm for 16 hr. The gradients were fractionated on an Isco Model 640 gradient fractionator. The absorbance of the RNA was measured by 254 nm. Fractions (0.6 ml) were collected directly into scintillation vials. Water (1.4 ml) and 10 ml of scintillation fluid was then added to each vial for measurement of radioactivity.

The electrophoresis of RNA was carried out on polyacrylamide gels (2.4%) for 180 min. The gels were scanned for absorbance with a Beckman Model 25 spectrophotometer equipped with a gel-scanning

attachment and finally sliced into 2 mm sections and placed in scintillation vials. The gel slices were incubated overnight at 37° in 0.5 ml of 0.5 N NaOH. Scintillation fluid was added and the samples counted in a Searle Mark III liquid scintillation counter.

To determine the nature of [^{14}C]Inox incorporation, the tumor cells were incubated in culture with [^{14}C]Inox (0.025 μCi/ml) the cells harvested and processed by the Schmidt-Thannhauser procedure. The acid-insoluble fractions were resolubilized in 2.0 ml of 0.02 M potassium phosphate buffer, pH 7.6. Sodium borohydride reduction of the incorporated [^{14}C]Inox was as described by Salvo *et al.* [20]. Following reduction the samples were dialyzed for 48 hr against 0.02 M potassium phosphate buffer, pH 7.6 with four buffer changes. The pH of the four samples was adjusted to 7.2 and enzyme added as follows: #1 control, none; #2 pronase, 2 mg/ml; #3 ribonuclease A, 1.0 mg/ml; #4 deoxyribonuclease, 1.0 mg/ml. The reactions were carried out at 37° for 24 hr and stopped by the addition of 0.16 ml of 60% TCA to each fraction and centrifugation at 15,000 rpm, 10 min. The pellet was resolubilized in 1.0 ml of 0.5 N NaOH.

In a separate set of experiments, Ehrlich tumor cells were incubated in culture with various concentrations of Inox (0.1, 0.5 and 1 mM, final concentrations). RNA was isolated from the control and Inox-treated cells by phenol extraction and ethanol precipitation. The RNA was dissolved in 0.5 ml of phosphate buffer, pH 7.6. [^{3}H]$NaBH_4$ (0.25 mCi/m-mole) was added to each sample and the reaction was carried out for 1 hr on ice. The reaction mixture was diluted to a final vol. of 1 ml and the RNA was precipitated by the addition of 2 ml of cold ethanol. The RNA pellet was isolated by centrifugation and the pellet washed with 75% ethanol/1% potassium acetate. The RNA was redissolved in potassium acetate and reprecipitated with ethanol. The RNA was finally collected by centrifugation and the RNA pellet dissolved in H_2O. The RNA concentration was determined by absorbance measurements at 260 and 280 nm.

The biochemicals used in these studies were purchased from Sigma Chemical Co.

RESULTS

In order to determine the distribution of Inox incorporation into Ehrlich ascites tumor cells, the cells were incubated in culture and labeled with [^{14}C]Inox. The incubation was carried out for 3 hr and incorporation into acid-soluble and acid-insoluble fractions was determined. [^{14}C]cytidine was added to control cultures as a measure of cellular activity. Table 1 shows the incorporation of [^{14}C]cytidine and [^{14}C]Inox into the tumor cells in culture. Incorporation of [^{14}C]cytidine in control culture indicated that the cells were very active in terms of metabolic activity. [^{14}C]Inox was incorporated into both acid-soluble and acid-insoluble pools of the cells.

Table 1. Incorporation of [^{14}C]Inox into Ehrlich tumor cells

Labeled nucleoside	Acid-soluble (total cpm/fraction)	Acid-insoluble (total cpm/fraction)
[^{14}C]cytidine*	114,400	165,200
[^{14}C]Inox†	42,900	23,400

* [^{14}C]cytidine (387 mCi/m-mole; 0.5 μCi/culture flask).
† [^{14}C]Inox (550 mCi/m-mole; 0.25 μCi/culture flask).

As a further control, to rule out the possibility of non-selective adsorption of the [^{14}C]Inox to the acid-insoluble fraction in the absence of incubation, tumor cells were put into culture as previously described. The cell pellet was isolated by centrifugation. [^{14}C]Inox (5.4×10^5 cpm) was then added to the cell pellet and the cell pellet was subjected to the Schmidt-Thannhauser procedure. There was absolutely no radioactivity in the acid-insoluble fraction indicating that there was incorporation or at least binding of the [^{14}C]Inox to the protein, RNA or DNA components of the acid-insoluble fraction in the incubated samples.

To determine the nature of the [^{14}C]Inox incorporation into the acid-soluble fraction, tumor cells were incubated for 1.5 and 3 hr with [^{14}C]Inox in culture. The acid-soluble fractions were isolated and pooled into two groups representing the 1.5 and 3.0 hr samples, respectively. The TCA from the acid-soluble fraction was removed by ether extraction and the samples were lyophilized. The samples were resuspended in 0.2 ml H_2O and 50 μl of each was placed on the HPLC as described in the methods section. Figures 1 and 2 show the elution profiles and radioactivity profiles superimposed for the 1.5 and 3.0 hr incubation, respectively. It can be seen that at 1.5 hr, there were two early peaks at 1.8 and 3.0 min respectively. Since both Inox and hypoxanthine are eluted at 1.8 min one cannot unequivocally attribute this first peak to Inox since it may represent degradation of the compound. The identity and significance of the 3.0 min peak will be discussed later. There were also peaks of radioactivity at the elution points of the 5′-mono-, di- and triphosphate nucleosides. The elution profile at 3.0 hr was very similar to that at 1.5 hr except there was a decrease in the radioactivity in the early peaks and an increase in the radioactivity

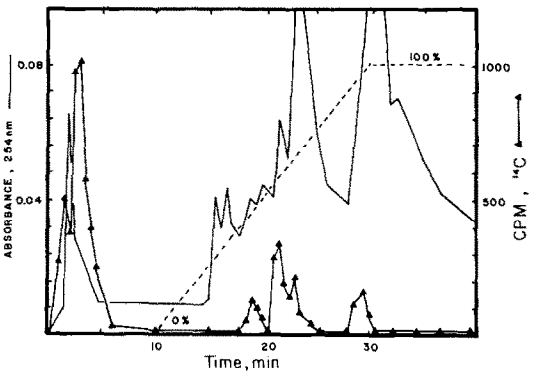

Fig. 1. High pressure liquid chromatography of acid-soluble fraction from [^{14}C]Inox-treated cells. The tumor cells were incubated for 1.5 hr in the presence of [^{14}C]Inox (0.25 μCi). The acid-soluble fraction was subjected to HPLC as described in the 'Methods' section. The u.v. absorbance is indicated by the solid line ——; the radioactivity by ▲——▲; and the gradient development by -----.

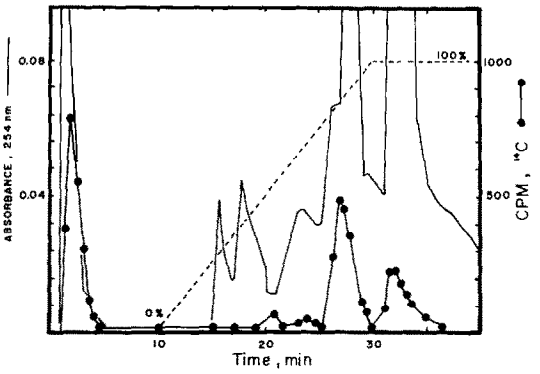

Fig. 2. High pressure liquid chromatography of acid-soluble fraction from [^{14}C]Inox-treated cells. The tumor cells were incubated for 3.0 hr in the presence of [^{14}C]Inox (0.25 μCi). The u.v. absorbance is indicated by the solid line ——; the radioactivity by ●——●; and the gradient development by -----.

found in the area of the 5'-di- and triphosphate nucleosides.

To determine whether the peak of radioactivity at 1.8 min corresponded to Inox or hypoxanthine, the 1.8 min fraction was collected and spotted on Whatman 3M paper and developed by descending chromatography with *n*-butanol–glacial acetic acid–H_2O (50:25:25). The Inox, PI-IMP and PI-ITP standards remained at the origin while hypoxanthine had a R_f of 0.50. It can be seen from Fig. 3 that the Inox derivatives were the primary species present.

To quantitate the change in the composition of the ^{14}C-labeled species in the acid-soluble pools, a portion of both 1.5 and 3.0 hr samples was applied to Dowex-1-formate columns and eluted as described. The distribution of radioactivity can be seen in Table 2 and these data agree very well with the elution profiles from HPLC. It can also be seen that the primary species at 3 hr was either the 5'-di- and/or triphosphate derivative of Inox and/or 5'-purine nucleotides.

As further controls, acid-soluble fractions were pre-

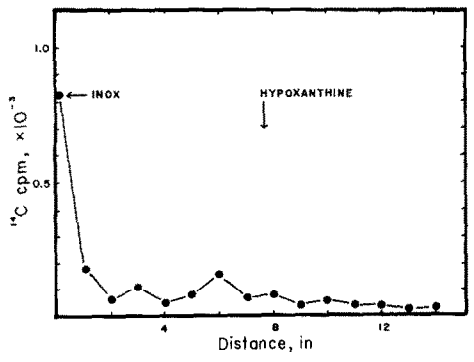

Fig. 3. Paper chromatography of Fraction after HPLC. The fraction eluting from the HPLC with the void vol. (1.8 min) was collected, concentrated and chromatographed on Whatman 3MM. The developing solvent was *n*-butanol–glacial acetic acid–H_2O (50:25:25). The chromatogram was cut-up into $\frac{1}{4}$ inch sections and counted directly for ^{14}C. The migration of the hypoxanthine standard is indicated by the arrow.

Table 2. Distribution of [^{14}C]Inox into acid-soluble pool of Ehrlich tumor cells

Eluant	Standards	% Total* 1.5 hr	3.0 hr
H_2O	Inox, purine nucleosides	34	14
2 N Formic Acid	Dialdehyde 5'-monophosphates purine 5'-monophosphates	13	24
3 N HCl	Dialdehyde 5'-di- and triphosphates purine 5'-di- and triphosphates	52	62

* The amount of radioactivity put over the Dowex-formate columns was 20,000 and 30,000 cpm from the acid-soluble fractions from the tumor cells incubated 1.5 and 3.0 hr, respectively.

pared from Ehrlich tumor cells as described in the "Methods" section. [^{14}C]Inox was added and the samples neutralized by ether extraction of the TCA. One group of samples (in triplicate) were put over the Dowex-1-formate columns immediately. The other group of samples were incubated at 37° for 2 hr prior to putting the samples over the Dowex-1-formate columns. The results of this experiment are shown in Table 3. It can be seen that even after 2 hr of incubation of [^{14}C]Inox with the components of the acid-soluble fraction only 5% of the radioactivity was eluted with HCl. This is far below the 52% and 62% seen for the distribution of [^{14}C]Inox compounds in the 1.5 and 3.0 hrs incubations (Table 2).

To determine whether Inox itself was being phosphorylated or whether the base was being reutilized, the 3.0 hr acid-soluble pool was fractionated on Dowex-1-formate and the HCl-eluant was collected. This was taken to dryness under vacuum and resuspended in 0.2 ml H_2O and then treated with *E. coli* alkaline phosphatase for 3.0 hr at 37°. The reaction was stopped by heating and 50 μl of the sample was injected into the HPLC and eluted with 0.1 M borate buffer. Any unreacted nucleotide would remain on the column and the released nucleoside or dialdehyde would be eluted. It can be seen from Fig. 4 that approximately 99% of the radioactivity was found as Inox after removal of the 5'-phosphate group.

A cell-free extract of Ehrlich ascites tumor cells was prepared and assayed for the presence of kinase activity with [^{14}C]Inox. All reactions were run at 37° for 1 hr and stopped with 0.5 ml of 5% TCA, the TCA was extracted and the samples fractionated over

Table 3. Binding of [^{14}C]Inox to components of acid-soluble fraction

	0 hr	2 hr
	cpm × 10^{-3}	
H_2O eluant	73.1 (89)*	82.7 (77)*
2 N formic acid	4.4 (5)	19.8 (18)
3 M HCl	4.4 (5)	5.6 (5)

* The numbers in parentheses are the % of radioactivity eluting in that fraction. The samples were set up in triplicate.

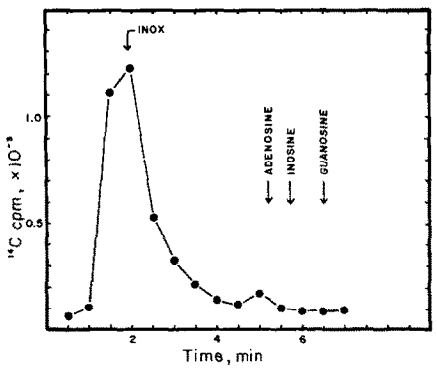

Fig. 4. HPLC of alkaline phosphatase-treated nucleotide pool after [^{14}C]Inox labeling. The acid-soluble fraction from [^{14}C]Inox-treated cells was treated with alkaline phosphatase as described in the 'Methods' section. This sample was then subjected to HPLC with 0.1 M sodium borate, pH 5 as the eluting buffer. The elution times of adenosine, inosine and guanosine are indicated by the arrows.

Dowex-1-formate. Table 4 shows that there was apparent conversion of [^{14}C]Inox to the 5'-monophosphate nucleotide and even greater conversion to the di- and triphosphate nucleotides.

It has been previously reported that the dialdehyde derivatives of nucleosides form stable complexes with the amino groups of proteins [2, 5, 14, 21]. We were also interested in whether Inox would form Schiff-bases with small mol. wt amino compounds, particularly amino acids. Inox (10^{-4} M) was incubated 2 hr at 37° with a 10-fold excess of glycine. Following incubation a sample was put on the HPLC and eluted with 0.1 M sodium borate buffer. The elution profile showed 2 peaks: 1.8 and 5.2 min, 1.9% and 98% of total respectively. Untreated Inox was eluted at 1.8 min. There is apparently efficient Schiff base formation of Inox and small mol. wt amino compounds. Returning to Fig. 1, it can be seen that the second major radioactivity peak probably represents such "Schiff-base" derivatives.

Table 4. Phosphorylation of [^{14}C]Inox by cell-free extract

Elution pool	cpm × 10^{-3} Control*	Sample*	Δ
H_2O	124.3 ± 5.3	108.9 ± 4.6	(−15.4)
2 M Formic acid	8.7 ± 0.7	9.8 ± 0.9	1.1
3 M HCl	8.9 ± 1.8	20.3 ± 2.4	11.4

* The recovery of radioactivity from the columns was 95 per cent for control and 94 per cent for the sample. All assays were carried out in triplicate. The data are presented with the standard deviations.

Table 5. Effect of nucleosides on the incorporation of [^{14}C]Inox into acid-soluble pools

	Total CPM incorporated	% Control
Control	20,412	—
Deoxyadenosine*	14,728	72
Deoxyguanosine	21,178	103
Adenosine	16,556	81
Cytidine	15,716	78
Guanosine	17,502	85
Inosine	17,822	87

* Concentration of the nucleosides was 0.1 mM. This concentration is a 2200-fold excess over [^{14}C]Inox.

Table 6. Effect of hypoxanthine on [^{14}C]Inox incorporation

	Total CPM	
	Acid-soluble	Acid-insoluble
Control	8825	3870
Hypoxanthine (0.1 mM)	6235 (70)*	2360 (61)*

* The values in parentheses are the % of control values. The culture flasks each contained 0.25 μCi [^{14}C]Inox.

The effect of nucleosides on the incorporation of [^{14}C]Inox into the acid-soluble fractions of the tumor cells is seen in Table 5. The nucleosides were added to the culture medium in 0.1 mM concentration and the incubations carried out for 2 hr. At this concentration the nucleosides would be in approximately 2200-fold excess over the [^{14}C]Inox. No significant effect was seen with any of the nucleosides tested which indicates that there was not a competitive transport mechanism for any of these nucleosides.

The effect of hypoxanthine on the incorporation of [^{14}C]Inox into the acid-soluble fractions was also tested with a 2200-fold excess of hypoxanthine in the culture medium. The data in Table 6 indicated that this concentration of hypoxanthine had only a small effect on [^{14}C]Inox incorporation into either acid-soluble or insoluble fractions. The effect of Inox on [^{14}C]hypoxanthine uptake by tumor cells is shown in Table 7. Again there was no significant effect. Together these data indicate that Inox was transported intact across the membrane and not degraded to hypoxanthine.

To determine whether the transport of Inox involved the ($Na^+ + K^+$)-ATPase system, the incorporation of [^{14}C]Inox into acid-soluble fraction of Ehrlich tumor cells was monitored in the presence of 0.1 mM and 1.0 mM oubain. There was no significant effect of oubain on Inox uptake at either concentration.

To determine the distribution of incorporation of [^{14}C]Inox into the components of the acid-insoluble fraction of Ehrlich tumor cells, the acid-insoluble fractions were subjected to selective enzymatic degradation after they had been labeled with [^{14}C]Inox. Four samples were prepared and processed as previously described, having control, pronase, RNase A and DNase I-treated fractions. From the data in Table

Table 7. Effect of Inox on [^{14}C]hypoxanthine incorporation

	Total CPM	% Control
Control*	938,493	100%
Hypoxanthine (0.1 mM)	119,808	12%
Inox (0.5 mM)	1,031,286	109%

* The culture flasks each contained 0.5 μCi of [8-^{14}C]hypoxanthine (52 mCi/m-mole).

Table 8. Enzymatic release of radioactivity from [^{14}C]Inox-labeled acid-insoluble fractions

Enzyme treatment	cpm Acid-soluble/ cpm Acid-insoluble*
none	
Pronase	0.30
RNAse A	0.36
DNAse	0.004

* The cpm acid soluble/cpm acid insoluble is the ratio of the radioactivity released by the treatment to the radioactivity remaining as acid-insoluble after the enzyme treatment.

8 it can be seen that the majority of the incorporated radioactivity was released as acid-soluble material by RNase and pronase with virtually no release by DNase. The incorporation into the protein fraction was expected since it has been previously shown that Inox forms stable Schiff-base complexes with the amino groups of proteins.

Cultures of Ehrlich tumor cells were again incubated for 2 hr with [^{14}C]Inox and the RNA was isolated by phenol extraction, washed and placed on 5–47% sucrose gradients and centrifuged at 26,000 rpm for 16 hr. The gradients were fractionated and the absorbance and radioactivity determined for each fraction. In Fig. 5, the u.v. absorbance pattern indicates the RNA was separated into 28S, 18S and small mol. wt species. The majority of the [^{14}C]Inox was found to be incorporated into the small mol. wt RNA species.

Actinomycin D (20 μg/ml) inhibited the incorporation of [^{14}C]Inox into RNA by 50 per cent as determined in the acid-insoluble fraction, or in the phenol-extracted RNA.

To confirm further that Inox was incorporated into RNA, tumor cells were incubated in culture with 0.1 mM, 0.5 mM and 1.0 mM Inox (unlabeled) in the culture media. The cells were harvested and the RNA isolated by the phenol method. The RNA was washed and resuspended in 0.02 M potassium phosphate buffer pH 7.6. Each sample was then treated with

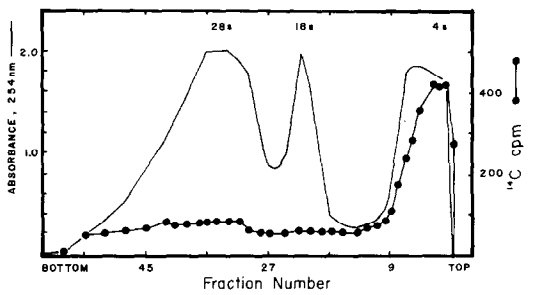

Fig. 5. Sucrose gradient fractionation of RNA from [^{14}C]Inox-treated cells. The phenol-extracted RNA was separated by sucrose gradient centrifugation. The gradient was fractionated by an Isco gradient fractionator connected to a u.v. monitor. The fractions were collected directly into scintillation vials. The u.v. absorbance at 254 nm is indicated by the solid line ——; the radioactivity by the closed circles ●—●.

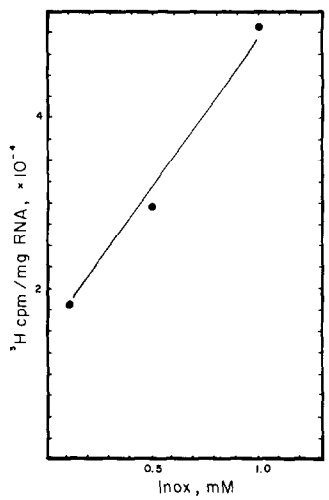

Fig. 6. Incorporation of ^{3}H into the RNA from Inox-treated cells following [^{3}H]NaBH$_4$ reduction. RNA was isolated by phenol extraction after treatment of the tumor cells with 0.1, 0.5 and 1.0 mM Inox. The isolated RNA was then treated with [^{3}H]NaBH$_4$. The data are plotted as the ^{3}H counts incorporated into the RNA from the Inox-treated cells above the ^{3}H counts incorporated into the RNA from the control cells.

[^{3}H]NaBH$_4$ (0.025 mCi) to reduce the dialdehyde groups to the dialchol and to label the Inox molecules. The reactions were carried out in the dark at 4° for 1 hr. After purification an aliquot of each sample was used for measurement of radioactivity while another aliquot was subjected to density gradient centrifugation on 5–47% sucrose gradients. From Fig. 6 it can be seen that the amount of ^{3}H incorporated above the control was approximately linear with the Inox concentrations used. Each sample (approx. 0.5 mg) was placed on sucrose gradients and fractionated as previously described. In Table 9 the results of fractionation indicate that again the primary area of Inox labeling was in the small mol. wt RNA and this labeling was a function of Inox concentration.

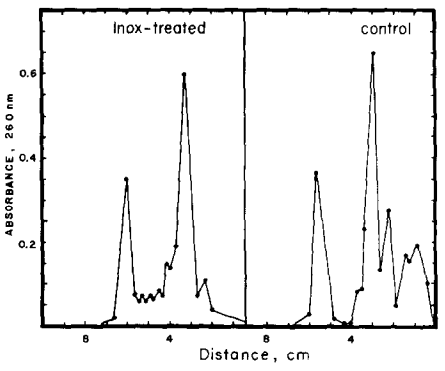

Fig. 7. Electrophoresis of RNA from control and Inox-treated mice *in vivo*. Mice were treated with Inox (25 mg/kg) for 2 days, at 5 days after tumor inoculation. Tumor cells were isolated from the control (untreated) and Inox-treated mice, and RNA isolated from these cells. The RNA was electrophoresed on polyacrylamide gel (2.4%) and scanned at 260 nm for u.v. absorption.

Table 9. Sucrose gradient fractionation of [^{3}H]NaBH$_4$ labeling of RNA from Inox treated cells

	Peak fractions from gradient CPM/mg		
	small M.W.	18S	28S
Control	83,200	9900	12,600
	CPM/mg above control		
0.1 mM Inox	800	—	—
0.5 mM Inox	11,900	—	—
1.0 mM Inox	31,600	—	5900

The effect of Inox on RNA *in vivo* was tested by injecting mice bearing Ehrlich tumor cells (5 days after inoculation) with 25 mg/kg/day on 2 successive days and then harvesting the cells. Control mice were also kept. Tumor cell counts were made for each group. RNA was isolated from these cells by the phenol method and the concentration of the RNA determined. There was approximately 70 per cent decrease in tumor cells after 2 days of treatment. There was a 30 per cent decrease in the total amount of RNA per cell. None of the treated or control mice died from the treatment with the drug. The RNA from tumor cells of both control and Inox-treated mice was subjected to gel electrophoresis on 2.4% polyacrylamide gels for 180 min. The gels were scanned for absorbance. Figure 7 shows the profile from control and Inox-treated mice. There was an apparent quantitative decrease in the ribosomal precursor RNA in the treated tumor cells, but no apparent qualitative differences were apparent after this particular treatment.

DISCUSSION

A study of the intracellular distribution and metabolism of Inox (NSC-118994) was undertaken because relatively little is currently known about its intracellular metabolism and in particular its actions on RNA. More importantly, Phase I clinical testing of this compound has been undertaken. Rational clinical use will necessitate further knowledge about all major sites of action as well as the intracellular metabolism of the compound. Should its use in combination chemotherapy protocols be undertaken, this information would also be of great use in choosing adjunctive agents.

Inox was shown to be well taken up by tumor cells without degradation (Table 1, Fig. 1). The exact mechanism of transport of the compound has not been elucidated but its transport is apparently not competitively linked to the transport of the major ribonucleosides or deoxyribonucleosides (Table 5). Its transport was not affected by oubain in millimolar concentrations indicating that its transport does not involve the ($Na^+ + K^+$)-ATPase system, but does not entirely rule out an energy-mediated transport.

Within the tumor cells, Inox is apparently stable. Inox was shown to be present in the nucleoside and nucleotide pools of the tumor cell (Table 2, Fig. 1, 2 and 3). Inox was apparently acted on by tumor cell kinases as there was a gradual increase in the incorporation of [^{14}C]Inox into nucleotide material with time within tumor cells (Fig. 1 and 2). Cell-free extracts from the Ehrlich ascites tumor cells was also demonstrated to have this activity (Table 4).

No significant level of radioactivity was released as acid-soluble material by treatment of the [^{14}C]Inox-labeled, acid-insoluble material with DNase, while radioactivity was released by either pronase or RNAase treatment. This would suggest that [^{14}C]Inox was not incorporated into the DNA of the tumor cells (Table 9). It would appear that at least, one of the sites of action of Inox on DNA synthesis was the inhibition of the ribonucleotide reductase step as previously reported [9, 10].

Inox has also been shown to form relatively stable Schiff-bases with large and small mol. wt amino compounds and a portion of the compound probably exists as the Schiff-base derivative of amino acids and proteins within the tumor cell.

Significant incorporation of Inox into the RNA fraction of the tumor cells was observed (Table 8). This incorporation was shown to occur primarily in the small mol. wt RNA species (Fig. 5). This incorporation of Inox into RNA appeared to be concentration dependent (Fig. 6, Table 9). Whether this incorporation into the small mol. wt RNA species was due to specific incorporation into the 4S and 5S RNA's or whether the incorporation into the small mol. wt species represented chain termination of large mol. wt RNA being synthesized is not known. However, since treatment with Inox was shown to cause a quantitative decrease in the precursor RNA species but no significant effect on 28 or 18S RNA (Fig. 7), it would appear that Inox has its greatest effect on precursor RNA synthesis. Actinomycin D inhibited the incorporation of [^{14}C]Inox into RNA.

Inox was also shown to be effective *in vivo* against Ehrlich ascites tumor cells when given intraperitioneally (5 days after tumor cell inoculation) at a dose of 25 mg/kg/day for two days. At this dose there was a 70 per cent reduction in tumor cells in two days.

Inox appears to be a potentially effective antitumor agent which has complex effects on RNA and DNA synthesis within tumor cells.

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