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Studies of Rhodium(II) Carboxylates as Potential Antitumor Agents[1] (37996)

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It has been reported that several platinum complexes exhibit antitumor activity (1–6). Also, recent reports indicate that certain platinum complexes bind to biological macromolecules (4–6) and inhibit the syntheses of DNA, RNA, and proteins *in vivo*. In this connection, the complexation of rhodium(II) with organic acids can form a "cage" structure (Fig. 1) which lends unusual stability to an otherwise unstable oxidation state of rhodium. Adducts form easily in the open axial positions of the rhodiums with electron-donating ligands. The formation of these adducts *in vivo* should be able to bring about inhibitions of biological processes. Survival studies with Swiss mice bearing the Ehrlich ascites tumor and BDF_1 mice bearing the L1210 ascites tumor showed that rhodium(II) acetate gave rise to significant increases in survival times, especially in combination with arabinosylcytosine (7). However, when used alone, rhodium(II) acetate was not particularly effective in the therapy of BDF_1 mice bearing the L1210 tumor (7), whereas Swiss mice carrying implants of the Ehrlich tumor responded well to the complex. In the studies reported here, we compare the antitumor activities of rhodium(II) acetate and rhodium(II) propionate and show that rhodium(II) propionate is a much more potent drug. In addition, we show that both compounds inhibit DNA and RNA polymerases and that the radioactive rhodium(II) acetate binds to several biological macromolecules, including DNA.

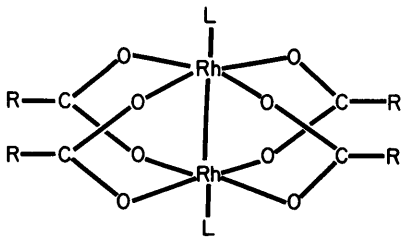

FIG. 1. The 6-coordinated cage complex of rhodium(II) carboxylates. *L* is the unfilled axial bond filled by an electron-donating atom. R = CH_3 = rhodium(II) acetate; R = CH_2CH_3 = rhodium(II) propionate.

Materials and Methods. Chemicals. Rhodium(II) acetate was purchased from Matthey Bishop, Inc., Malvern, PA 19355. The other rhodium(II) carboxylates were synthesized by methods previously described (8). The radiolabeled rhodium(II) acetate was synthesized by ligand exchange with 1-^{14}C-acetic acid (New England Nuclear) by the method of Johnson *et al.* (8) except that the radioactive organic acid was employed. Polyriboadenylate (poly A) polyriboguanylate (poly G), polyribocytidylate (poly C), calf thymus DNA, and bovine serum albumin were purchased from the Sigma Chemical Company. Bovine pancreatic ribonuclease A (RNase) was obtained from the Worthington Biochemical Company.

Studies in vivo and in vitro. Female Swiss albino mice (Sprague–Dawley, Madison,

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WI) were each implanted ip with 4×10^6 Erlich ascites tumor cells and on day 7 after implantation, the cells were harvested. For *in vitro* whole cell experiments, tumor cells were removed with Pasteur pipets after laparotomy, the ascites fluid was removed by centrifugation at 1490*g* for 2 min in an International clinical centrifuge. To lyse red blood cells, the packed tumor cells were resuspended in 5 vol of ice-cold distilled water for 20 sec and then 5 vol of 1.8% saline were added and the contents thoroughly mixed. The cells were washed with 0.9% saline once more and diluted with medium. For enzyme experiments, cell-free extracts were prepared as follows: Washed cells free of red blood cells were suspended in 4 vol of ice-cold double-distilled water in the cold for 5 min and then homogenized in a Potter–Elvehjem homogenizer with 30 strokes. The cell particulates were centrifuged down in a Beckman J-21 centrifuge at 11,000 rpm (9500*g*) for 60 min at 4°C. The supernatant fluids were used for the enzyme assays. One milliliter of cell-free extracts from Ehrlich ascites cells contained 2.5–7.4 mg of protein. The protein concentrations were measured by the method of Lowry *et al.* (9).

Measurement of DNA synthesis. For *in vitro* whole-cell studies, the cells were suspended (25 mg wet weight per ml medium) in Fischer's medium. Drugs and cells were preincubated for 10 min at 37°C with shaking in air before the addition of TdR-methyl-^{3}H (5 μCi/μmole) in a final volume of 10 ml. At the specified times, 1-ml aliquots were delivered into 0.1 ml of 50% TCA. The precipitates were washed four times with 5 ml each time of ice-cold 5% TCA in the cold, and twice with 5 ml each time of ice-cold 95% ethanol. The precipitates were partially dried and resuspended in 1.5 ml of Protosol (New England Nuclear), and solubilized for 4 hr at 55°C. The samples were counted in 10 ml of toluene scintillation fluor in a Packard Model 3380 liquid scintillation spectrometer.

DNA polymerase(s) assay. The assay method for DNA polymerase activity was described previously (10).

RNA polymerase(s) assay. The assay for RNA polymerase(s) activity was also previously described (11).

Binding studies. Rhodium(II) acetate-1-^{14}C was dissolved in 0.01 *M* Tris–HCl, pH 7.2, and 0.02 *M* NaCl to give a final concentration of 10^{-4} *M*. Each dialysis sack contained 0.5 ml of either native calf thymus DNA (2 mg/ml), denatured calf thymus DNA (2 mg/ml), ribonuclease A (2 mg/ml), albumin (2 mg/ml), polyriboadenylic acid (2 mg/ml), polyriboguanylic acid (2 mg/ml), or polyribocytidylic acid (2 mg/ml). The dialyses were carried out at 37°C. The ratios of the sample solution in the dialysis sacks to the rhodium(II) acetate solutions in the dialysis baths were 1:100. At the indicated times, each dialysis sack was taken out and an 0.1-ml aliquot was mixed with 5 ml Aquasol (New England Nuclear) and counted.

Inhibition of de novo purine biosynthesis. Henderson's (12) method was employed to measure inhibition of the *de novo* purine biosynthetic pathway.

Survival studies. Female Swiss albino mice were each implanted ip with 4×10^6 Ehrlich ascites cells. Drug treatment was started 24 hr after implantation and injections were given ip once daily for 6 days. Drugs were dissolved in saline and given in a volume of 0.2 ml. Control mice received saline only.

Toxicity studies. In order to ascertain the proper dosages of rhodium(II) acetate and rhodium(II) propionate for use in the therapy of tumor-bearing mice, the method of Skipper *et al.* (13) was used to determine the LD_{50} and LD_{10} doses for Swiss mice.

Results. Toxicity studies. The LD_{10} and LD_{50} of rhodium(II) acetate were found to be 19 mg/kg and 27 mg/kg, respectively. For rhodium(II) propionate, the LD_{10} and LD_{50} were 2.7 mg/kg and 4.5 mg/kg, respectively. In survival studies, rhodium(II) acetate was given at 16 mg/kg and rhodium(II) propionate at 2.0 mg/kg.

Survival studies. The results of survival studies with female Swiss mice bearing the Ehrlich ascites tumor are shown in Table I. Rhodium(II) acetate at 16 mg/kg given once daily for 6 days, and rhodium(II)

TABLE I. Survival Study of Swiss Mice Bearing the Ehrlich Ascites Tumor.[a]

	Drug	Dosage (mg/kg × daily treatments)	Survival time (days ± SE)	Number of 50-day survivors
Expt I	Saline		14.1 ± 0.6	—
	Rhodium (II) acetate	16 × 6	30.3 ± 2.9	—
Expt II	Saline		15.3 ± 0.9	—
	Rhodium (II) acetate	16 × 6	30.1 ± 4.1	—
Expt III	Saline		17.2 ± 0.5	—
	Rhodium (II) propionate	2 × 6	33.1 ± 2.7	1
Expt IV	Saline		16.0 ± 0.7	—
	Rhodium (II) propionate	2 × 6	35.9 ± 3.1	3

[a] Female Swiss mice were each implanted ip with 4×10^6 Ehrlich ascites cells, and drug therapy was started 24 hr later. Each group contained 10 mice. Survival studies were carried out for 50 days. Mice surviving 50 days were calculated as surviving only 50 days. Drugs were dissolved in saline and given ip once daily for 6 days.

propionate at 2 mg/kg given once daily for 6 days, both doubled the survival times of the tumor-bearing mice when compared with the controls.

Studies in vitro. The effects of rhodium(II) acetate and rhodium(II) propionate at 10^{-5} *M* on the incorporation of TdR-^{3}H into the DNA of Ehrlich ascites cell suspensions are shown in Fig. 2. At 10^{-5} *M*, rhodium(II) acetate gave minimal inhibition to TdR incorporations into DNA, while rhodium(II) propionate gave about an 80% inhibition.

DNA and RNA polymerases. Table II shows the inhibitory effects of the two rhodium(II) carboxylates on the DNA polymerase(s) of the Ehrlich ascites tumor. At 5×10^{-5} *M* concentrations, rhodium(II) propionate gave almost complete inhibition and rhodium(II) acetate, about one-half this amount. Arabinosylcytosine (ara-C) was used here as the inhibitory control. At the same inhibitory molarity (5×10^{-5} *M*) rhodium(II) acetate did not significantly block RNA synthesis (Table III) while rhodium(II) propionate decreased RNA synthesis only about 40%.

Inhibition of the de novo purine pathway. Rhodium(II) propionate apparently produced potent inhibition of the *de novo*

TABLE II. Inhibition of DNA-Dependent DNA Polymerase(s) of Ehrlich Tumor Cells.[a]

Drugs	Concentration (*M*)	dTTP incorporated (pmoles)	Inhibition (%)
Control		260	0
Ara-C	5×10^{-5}	28	89
Rhodium (II) acetate	5×10^{-5}	139	47
Rhodium (II) propionate	5×10^{-5}	19	93

[a] Reaction tubes, in duplicate and complete except for deoxyribonucleoside triphosphate substrates, were preincubated with drugs or water for 5 min at 37°C. The reaction tubes each contained 50 μmoles of Tris–HCl, pH 8; 5 μmoles of $MgCl_2$; 100 μg of denatured calf thymus DNA; 75 μg of creatine phosphokinase; 5 μmoles of creatine phosphate; 1 μmole of ATP; 150 μl of cell-free extract (1.10 mg of protein) rhodium(II) carboxylates of ara-C; and 10 nmoles each of dATP, dGTP, dCTP, and dTTP-methyl-^{3}H (25 μCi/μmoles) in a final volume of 0.500 ml. After preincubation with drugs or water, incubation was continued for another 15 min at 37°C after the addition of substrates.

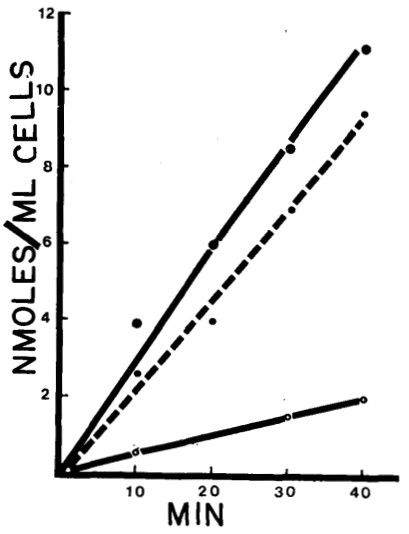

FIG. 2. *In vitro* inhibition of thymidine-^{3}H incorporation into Ehrlich ascites cell suspensions by rhodium(II) carboxylates. (●—●) Control; (●- - -●) 10^{-5} *M* rhodium(II) acetate; (○—○) 10^{-5} *M* rhodium(II) propionate. The incubation mixture contained in Fischer's medium, pH 7.5, the following: thymidine-^{3}H, 5×10^{-4} *M*, 5 μCi/μmole; rhodium(II) acetate or rhodium(II) propionate, 10^{-5} *M*; 25 mg wet weight of cells per ml of medium; in a final volume of 10 ml in 50-ml Ehrlenmeyer flasks. Incubations were carried out in air in a metabolic shaker at 38°C. The rhodium(II) carboxylates, dissolved in medium, were preincubated with cells in medium for 10 min at 38°C before adding the thymidine-^{3}H. At designated times, 1.0-ml aliquots were removed for analysis.

purine pathway at 10^{-4} *M* (Table IV) while rhodium(II) acetate was without appreciable effect at that concentration. Methylthioinosine used as the inhibitory control at 10^{-4} *M* produced an 80% inhibition; about the same degree of metabolic blockade as 10^{-4} *M* rhodium(II) propionate.

Binding studies. Rhodium acetate-1-^{14}C was found to bind to denatured DNA and (poly A), but not to (poly G), (poly C) or native DNA (Fig. 3). In other studies not depicted, bovine pancreatic ribonuclease A and bovine serum albumin were also found to bind well with rhodium acetate.

Discussion. The antitumor activity of rhodium(II) acetate was corroborated in our present study. In addition, the next higher homolog, rhodium(II) propionate, was found to be somewhat more potent as an anticancer agent—by a factor of about 10 on a molar basis (16 mg/kg of rhodium(II) acetate equals 38 μmoles/kg, and 2 mg/kg, of rhodium(II) propionate equals 4 μmoles/kg). These results were approximated by the inhibitions of thymidine incorporations into DNA. However, these comparisons of the two rhodium(II) carboxylates as regards inhibitions of RNA and DNA polymerase(s) did not hold up in that at equimolar concentration the rhodium(II) propionate was only about 2-fold more effective an inhibitor. Since crude enzyme extracts were used, any such com-

TABLE III. Inhibition of DNA-Dependent RNA Polymerase (s) of Ehrlich Tumor Cells.[a]

Drugs	Concentration (*M*)	UTP incorporated (pmoles)	Inhibition (%)
Control		76.7	0
Rhodium (II) acetate	1×10^{-5}	71.1	7
Rhodium (II) acetate	5×10^{-5}	65.4	15
Rhodium (II) propionate	1×10^{-5}	61.7	20
Rhodium (II) propionate	5×10^{-5}	46.4	40

[a] Reaction tubes, in duplicate and complete except for nucleoside triphosphate substrates, were preincubated for 5 min at 37°C. The reaction tubes each contained 50 μmoles of Tris–HCl, pH 7.9; 2.5 μmoles of $MgCl_2$; 2.5 μmoles of $MnCl_2$; 10 μmoles of NaF; 100 μg of calf thymus native DNA; various concentrations of rhodium (II) carboxylates or water; 200 nmoles each of ATP, GTP, CTP, and UTP-5-^{3}H (sp act 10 μCi/μmole) and 0.150 ml of cell-free extract (1.10 mg of protein) in a final volume of 0.500 ml. Incubation was continued for another 10 min after the addition of substrates.

TABLE IV. Inhibition of Formylglycinamide Ribonucleotide Accumulation in Ehrlich Tumor Cells.[a]

Drugs	Concentration	FGAR[b] accumulated (CPM)	Inhibition (%)
Control		55,071	0
Methylthioinosine	1×10^{-4}	10,741	81
Rhodium (II) acetate	1×10^{-5}	48,099	13
Rhodium (II) acetate	1×10^{-4}	42,340	23
Rhodium (II) propionate	1×10^{-5}	22,301	60
Rhodium (II) propionate	1×10^{-4}	12,132	88

[a] Ehrlich tumor cells (25 mg wet weight/ml of medium) were suspended in Krebs–Ringer (without $CaCl_2$) phosphate buffer, pH 7.4, containing 5.5 m*M* glucose. Reaction flasks, in duplicate and complete except for glutamine and glycine-2-^{14}C, were preincubated for 5 min at 37°C. Each reaction flask contained 25 mg (wet weight) of tumor cells; 4 μg of azaserine; various concentrations of drugs; 2 μmoles of L-glutamine; and 1 μmole of glycine-2-^{14}C (sp act 1 μCi/μmole) in a final volume of 2 ml. Incubation was continued for another 55 min after the addition of glutamine and glycine-2-^{14}C.

[b] FGAR, formylglycinamide ribonucleotide.

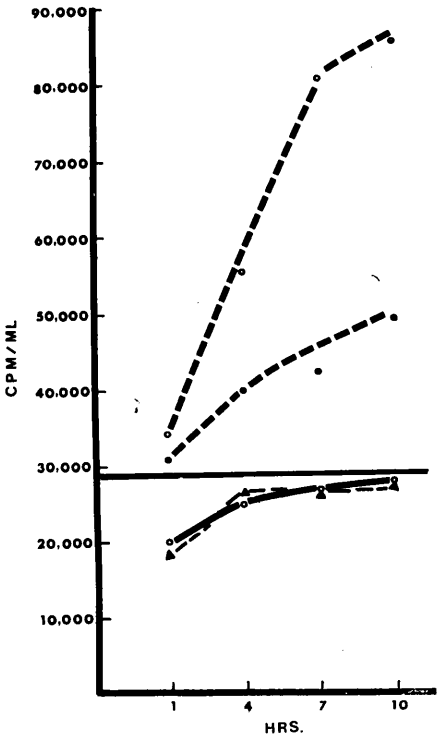

FIG. 3. Binding of rhodium(II) acetate-1-^{14}C to several macromolecules as determined by equilibrium dialysis. (○--○) Poly A; (●--●) Denatured DNA; (○—○) Poly G = Native DNA; (▲--▲) Poly C. See Methods and Materials for experimental protocol.

parisons may not be entirely valid and alternative interpretations should and will be developed at a later date. It appeared, however, that both rhodium(II) carboxylates were better inhibitors of DNA polymerase(s) than RNA polymerase(s) *in vitro*. In addition, rhodium(II) propionate inhibited *de novo* purine biosynthesis. In preliminary experiments (unpublished results), the rhodium(II) carboxylates were found to bind to free amino acids. It must be recalled that glycine and glutamine are required in the initial reactions prior to the formation of formylglycinamide ribonucleotide (FGAR).

An *in vitro* study has shown that *cis*-platinum(II) diaminodichloride will cross link complementary strands of DNA (6). Recently, Howle *et al.* (4) observed that *cis*-dichloro(dipyrimidine)-platinum(II) was able to bind to calf thymus DNA and RNA *in vitro*, but no appreciable binding to proteins was observed. To understand the interactions between rhodium(II) carboxylates (14) and various biological molecules, several questions were posed. One was whether bonding took place at one or both axial positions on the rhodium(II) complex; or did substitution for one or more of the carboxylate ions occur? An-

other was what types of molecules would form complexes with the rhodium(II) carboxylates and for those that bond, what donor atom was involved? To answer the last question, we studied the binding of rhodium(II) acetate to several macromolecules using equilibrium dialysis. As was seen in Fig. 2, binding of rhodium acetate-1-^{14}C to native calf thymus DNA did not seem to occur. However, binding was observed when calf thymus DNA was denatured. In order to ascertain what electron-donating atoms were responsible for binding of rhodium(II) acetate, the dialysis equilibrium experiments were undertaken with several homopolymers, such as polyriboadenylate (poly A), polyriboguanylate (poly G), and polyribocytidylate (poly C). Rhodium(II) acetate appeared to bind only to (poly A) under the experimental conditions employed. We tentatively conclude that the adenine preferentially interacted with rhodium(II) acetate.

Summary. Rhodium(II) acetate and rhodium(II) propionate were found to be antineoplastic agents. Both compounds inhibited DNA and RNA synthesis. The rhodium(II) propionate was also found to block *de novo* purine biosynthesis. Binding studies employing the carbon-14-labeled rhodium(II) acetate showed binding to DNA (poly A), and two proteins.

1. Rosenberg, B., Van Camp, L., Trosko, J. E., and Mansour, V. H., Nature (London) **222**, 385 (1969).

2. Rosenberg, B., and Van Camp, L., Cancer Res. **30**, 1799 (1970).

3. Gale, G. R., Howle, J. A., and Walker, E. M., Cancer Res. **31**, 950 (1971).

4. Howle, J. A., Gale, G. R., and Smith, A. B., Biochem. Pharmacol. **21**, 1464 (1972).

5. Connors, T. A., Jones, M., Ross, W. C. J., Braddock, P. D., Khokhar, A. R., and Tobe, M. L., Chem. Biol. Interactions **5**, 415 (1972).

6. Roberts, J. J., and Pascoe, J. M., Nature (London) **235**, 282 (1972).

7. Hughes, R. G., Jr., Bear, J. L., and Kimball, A. P., Proc. Amer. Ass. Cancer Res. **13**, 120 (1972).

8. Johnson, S. A., Hunt, H. R., and Neumann, H. M., Inorg. Chem. **2**, 960 (1963).

9. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem. **193**, 265 (1951).

10. Hughes, R. G., Jr., and Kimball, A. P., Cancer Res. **32**, 1791 (1972).

11. Spoor, T. C., Evans, J. E., and Kimball, A. P., Proc. Soc. Exp. Biol. Med. **136**, 605 (1971).

12. Henderson, J. F., J. Biol. Chem. **237**, 2631 (1962).

13. Skipper, H. W., Schmidt, L. H., Cancer Chemother. Rep. **17**. Part 1, (1962).

14. Kitchens, J., and Bear, J. L., J. Inorg. Nucl. Chem. **31**, 2415 (1969).

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