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A PROPOSED MODE OF ACTION OF ANTITUMOR PLATINUM COMPOUNDS BASED UPON STUDIES WITH *cis*-DICHLORO-([G-^{3}H]DIPYRIDINE)PLATINUM(II)*

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Abstract—The neutral, square-planar platinum complex *cis*-dichloro(dipyridine)platinum(II) (*cis*-PPC), which is a structural analog of *cis*-dichlorodiammineplatinum(II) (*cis*-PtII) and possesses similar antitumor and antimitotic properties, was synthesized using [G-^{3}H]pyridine and used to determine certain characteristics of its interactions with selected living and non-living systems. *Cis*-dichloro([G-^{3}H]dipyridine)platinum(II) (*cis*-PPC-^{3}H) associates with intact Ehrlich ascites tumor cells *in vitro* at 2° and 37° and resists dissociation by washing with saline or trichloroacetic acid (TCA) as well as solubilization in alkali followed by reprecipitation with TCA. Dialysis experiments showed that *cis*-PPC-^{3}H associates avidly with calf thymus DNA, high molecular weight yeast RNA, and bacterial and yeast transfer RNA, but not with bovine serum albumin, dextran, or purified erythrocyte membranes. Dialysis of the platinum–nucleic acid complexes in distilled water or NaCl results in loss of a portion of the original radioactivity. The platinum–DNA bond is also resistant to dissociation by solubilization in alkali followed by TCA precipitation. The homopolymers, polyadenylate and polycytidylate, bind *cis*-PPC-^{3}H to the same extent as RNA on a weight basis; polyguanylate and polyuridylate bind the platinum complex to a greater extent than does RNA. Similar to nitrogen mustard (HN_2), bonding of *cis*-PPC-^{3}H to DNA *in vitro* is inhibited by NaCl; however, prior alkylation of the DNA with HN_2 does not influence its subsequent bonding with *cis*-PPC-^{3}H. It was proposed that the antitumor and antimitotic action of the square-planar platinum complex depends upon the dissociation of one or both chlorine atoms from the platinum atom. The resulting cationic, aquated species subsequently forms a bond with nucleic acid, and does so with a possibly greater affinity for guanylate and uridylate. The site of establishment of this bond(s) is probably dissimilar to that of the HN_2–nucleic acid bond.

SINCE Rosenberg's[1] observation of the inhibitory effect of certain neutral platinum complexes on cell division of *Escherichia coli*, one of these compounds, *cis*-dichlorodiammineplatinum(II) (*cis*-PtII), has been shown to be a potent inhibitor of DNA synthesis in mammalian cells *in vitro* and *in vivo*.[2,3] Howle and Gale[2] suggested that *cis*-PtII may be pharmacologically inert as such, but undergo a transformation to an active species. Based upon intramolecular spatial considerations, Harder[3] proposed that platinum compounds exert an antitumor action by binding to DNA in a manner similar to that of nitrogen mustard (HN_2), and speculated that the target sites may be adjacent intrastrand purine residues.

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A recent report from this laboratory[4] described the antitumor and antimitotic properties of *cis*-dichloro(dipyridine)platinum(II) (*cis*-PPC) as well as its effects on nucleic acid and protein synthesis. This organic analog of the totally inorganic *cis*-PtII was found to be pharmacologically similar to *cis*-PtII in all systems investigated, albeit somewhat less potent. The present report describes the interactions of a radioactively labeled platinum antitumor agent, *cis*-dichloro([G-^{3}H]dipyridine)platinum(II) (*cis*-PPC-^{3}H) in certain living and non-living systems.

MATERIALS AND METHODS

Cis-PPC-^{3}H was synthesized by the method of Kauffman,[5] using potassium tetrachloroplatinate (a generous gift from Matthey Bishop, Inc.) and [G-^{3}H]pyridine (Amersham/Searle; specific activity, 1·9 mc/m-mole). Elemental analyses (Galbraith Laboratories) yielded data which agreed with theoretical values within 0·1 per cent, and the product had a specific activity of 3·2 mc/m-mole.

Ehrlich ascites carcinoma cells were maintained in BALB/c mice (Flow Research Animals, Inc.). Cells were harvested just prior to use, washed free of ascitic fluid, and suspended to 1% (v/v) in Eagle's minimum essential medium with Hanks' balanced salt solution, 0·002 M with respect to glutamine. The cells were then incubated with *cis*-PPC-^{3}H for various times, after which the samples were centrifuged and the cells were washed in accordance with each experimental design.

Materials for dialysis experiments included regenerated cellulose tubing (0·25 in., Arthur H. Thomas Company); calf thymus DNA, yeast RNA, polyadenylic acid,

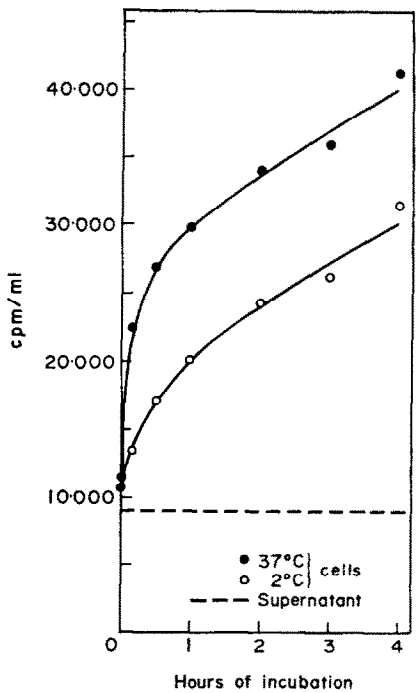

FIG. 1. Accumulation of *cis*-PPC-^{3}H into Ehrlich ascites tumor cells *in vitro* as a function of time at 37° and 2°. The cells were 1% (v/v) and *cis*-PPC-^{3}H was 3×10^{-5} M.

polycytidylic acid, polyguanylic acid, polyuridylic acid and crystalline bovine serum albumin (Nutritional Biochemicals Corp.); *E. coli* B tRNA and baker's yeast tRNA (Schwarz/Mann); dextran (mol. wt., 204,000; Sigma); and human erythrocyte membranes prepared as described by Augustinsson.[6] [1,2-^{14}C]HN_2 was purchased from Mallinckrodt Nuclear; nonradioactive HN_2 free of NaCl was generously donated by Merck, Sharpe & Dohme. Aliquots of 0·5 ml of a 0·05 per cent (except as otherwise indicated) polymer solution or membrane suspension were added to glycerol-free dialysis sacks. The open ends of the sacks were then closed and the preparations were placed into a solution of *cis*-PPC-^{3}H at 3×10^{-5} M. All dialysis experiments were done at 25°. The ratio of the volume outside the sack to that inside was 100 in all cases. Radioactivity was measured with a liquid scintillation spectrometer (Nuclear-Chicago, Mark I) with an efficiency of about 40 per cent.

RESULTS

Upon incubation in the presence of 3×10^{-5} M *cis*-PPC-^{3}H for a period of 4 hr at 37° followed by a single washing with 0·9% NaCl, there was a greater amount of radioactivity associated with a given packed volume of Ehrlich ascites tumor cells than with the same volume of culture medium. An accumulation of radioactivity was

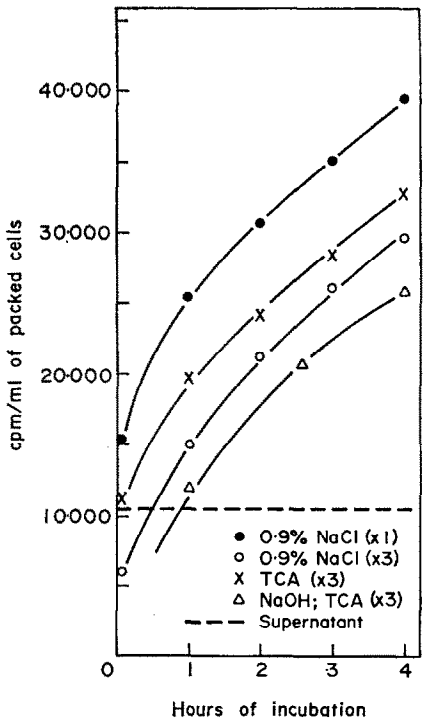

FIG. 2. Retention of *cis*-PPC-^{3}H in Ehrlich ascites tumor cells after treatment by the following procedures: gentle resuspension in 0·9% NaCl (× 1; closed circles); thorough washing by resuspension in 0·9% NaCl and centrifugation (× 3; open circles); precipitation with 5 per cent trichloroacetic acid and washing by centrifugation and resuspension (× 3; crosses); precipitation with trichloroacetic acid, solubilization in dilute NaOH, and reprecipitation with trichloroacetic acid followed by washing (× 3; triangles). *Cis*-PPC-^{3}H was 3×10^{-5} M and the temperature was 37° during incubation.

also evident in cells incubated at 2°, but to a lesser extent (Fig. 1). The accumulated radioactivity at the end of 4 hr of incubation at 37° corresponded to $4{\cdot}3 \times 10^{-4}$ $\mu\mu$mole of platinum/cell. The tenacity of the alliance was evinced by the fact that a considerable portion of that which was associated was resistant to dissociation upon repetitive washing with 0·9% NaCl, precipitation and washing with 5% trichloroacetic acid (TCA), or solubilization in 0·1 N NaOH followed by reprecipitation with TCA (Fig. 2).

The accumulation of radioactivity associated with the cells could occur if: (1) *cis*-PPC-^{3}H were being transported actively into the cell; (2) the compound were becoming attached to the surface of the cell; or (3) it were passively diffusing into the cell and being firmly bound to some intracellular constituent. Since lowering the incubation temperature to 2° did not obliterate the accumulation of radioactivity, and since the radioactivity remained associated with the TCA-insoluble fraction of the cell after repetitive washings, it was considered that binding of *cis*-PPC-^{3}H to some macromolecular constituent of the cell was the most probable basis of the accumulation of radioactivity.

To assess the degree of binding in a non-living system, preliminary experiments with a DNA solution enclosed in a dialysis sack immersed in a solution of *cis*-PPC-^{3}H were done in 5×10^{-4} to 5×10^{-2} M tris buffer at pH 7·0. At the lowest buffer concentration, there was some net accumulation of radioactivity into the DNA-containing sacks, but not at the higher concentrations. Since the pH was the same in all

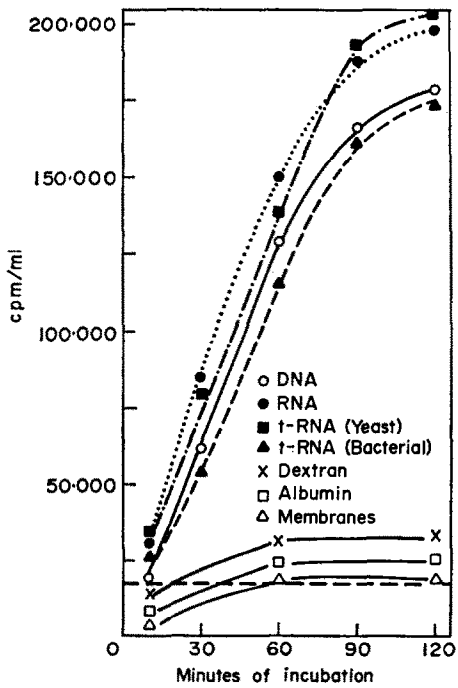

FIG. 3. Accumulation of *cis*-PPC-^{3}H across a semipermeable membrane into aqueous solutions of certain polymers and an aqueous suspension of erythrocyte membranes (0·05 per cent). The broken line near the bottom of the figure represents the activity of *cis*-PPC-^{3}H in the dialysis bath. Concentration of *cis*-PPC-^{3}H, 3×10^{-5} M.

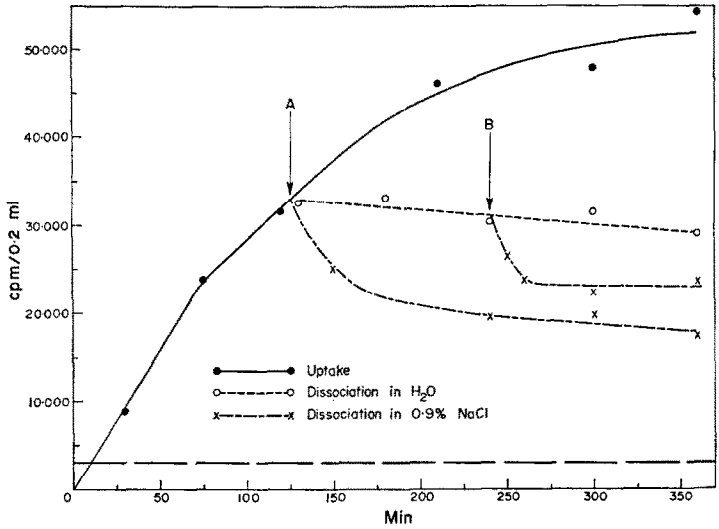

FIG. 4. Accumulation of *cis*-PPC-^{3}H into a DNA solution and its subsequent efflux in response to various conditions. The closed circles represent the accumulation of radioactivity over a period of 360 min into sacks of DNA solution immersed in a solution of *cis*-PPC-^{3}H, 3×10^{-5} M. At point A, a number of sacks were removed and placed into either distilled water (open circles) or 0·9% NaCl (crosses) and the efflux was monitored. At point B, several sacks were transferred from the distilled water bath to 0·9% NaCl and further efflux was measured. The broken line near the bottom of the figure represents the activity of *cis*-PPC-^{3}H in the initial dialysis bath.

flasks, this reduced accumulation at higher buffer concentrations was considered to be due most probably to a reduction by the tris ion of the extent of dissociation of the neutral platinum complex.[7]

Subsequent experiments were done in distilled water without buffer. In these procedures, the pH remained between 6·0 and 8·0. After 2 hr of dialysis in such a system, *cis*-PPC-^{3}H concentrated into the solutions of nucleic acids (highly polymerized calf thymus DNA, high molecular weight yeast RNA, *E. coli* tRNA and

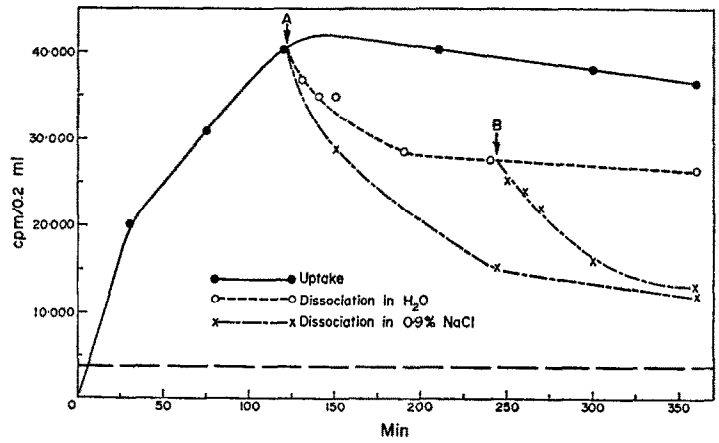

FIG. 5. Same as Fig. 4, except that RNA was substituted for DNA.

baker's yeast *t*RNA) up to a 10-fold extent, but only to a very moderate degree into solutions of dextran or albumin, or suspensions of erythrocyte membranes (Fig. 3). After 2 hr of dialysis, there was a diminution of the rate of accumulation of platinum across the membrane, indicating virtually complete saturation of the receptor sites on the nucleic acid molecule. Using the mean A + T/G + C ratio as given for calf thymus DNA,[8] 1 g DNA was found to bind 30 μmoles of platinum at the end of the 2-hr period, or approximately 1 atom of platinum per 2 nucleotides.

Of the *cis*-PPC-^{3}H accumulated into the DNA and RNA solutions during a 2-hr dialysis period, only a portion of it could be removed by further dialyzing the platinum-bound macromolecules in distilled water or 0·9% NaCl (Figs. 4 and 5). In addition, only incomplete dissociation was effected by repetitive precipitation with 5% TCA and solubilization in 0·1 N NaOH (Fig. 6).

Compounds of the type $PtCl_2A_2$, where A_2 is two ammine ligands, two amine ligands, or the bidentate ethylenediamine ligand, are known to lose both chloride atoms in aqueous solution in successive aquation reactions.[7] If this loss of chloride is essential for the interaction of platinum with nucleic acids, increasing the chloride concentration in the *cis*-PPC-^{3}H solution should retard this interaction. Dialysis sacks containing DNA (0·5 mg/ml) were thus incubated in solutions containing *cis*-PPC-^{3}H

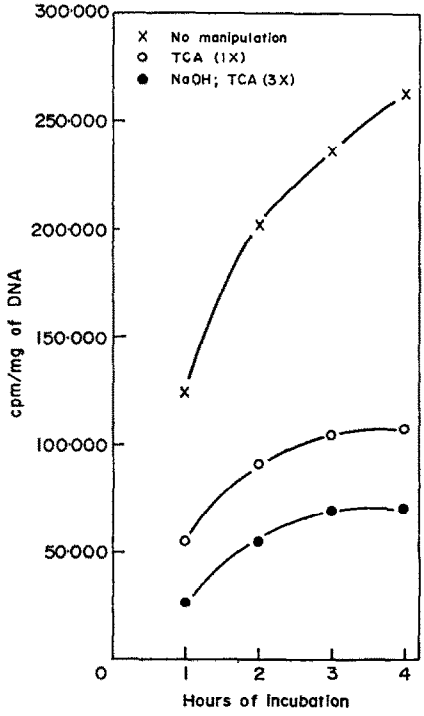

FIG. 6. Resistance of the DNA–platinum bond to dissociation by alternately precipitating with cold 5% TCA, sedimenting, and resolubilizing in 0·1 N NaOH. Concentration of *cis*-PPC-^{3}H, 3×10^{-5} M. Control values (crosses) were obtained by simply removing an aliquot of the solution from the dialysis sack and adding it directly to the scintillation phosphor. The open circles represent values obtained after a single precipitation with TCA; the closed circles represent values obtained after three repetitions of precipitation, sedimentation and resolubilization.

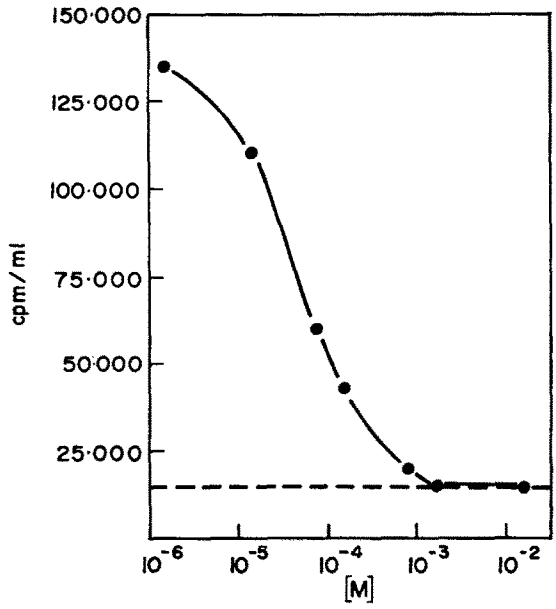

FIG. 7. Effects of graded concentrations of NaCl in water on the accumulation of *cis*-PPC-^{3}H across a semipermeable membrane into solutions of DNA. The dialysis baths contained *cis*-PPC-^{3}H at 3×10^{-5} M and NaCl as indicated on the abscissa. The broken line represents the activity of *cis*-PPC-^{3}H in the dialysis bath.

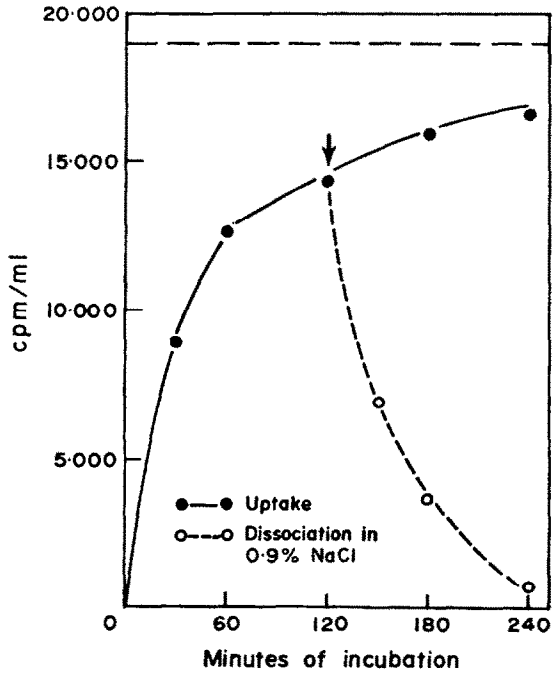

FIG. 8. Inhibition by NaCl (0·9%) of nondialyzable fixation of *cis*-PPC-^{3}H (3×10^{-5} M) to DNA. Closed circles represent the accumulation of *cis*-PPC-^{3}H into the sacks of DNA in the presence of NaCl. At the point indicated by the arrow, several sacks were transferred to a bath of 0·9% NaCl without *cis*-PPC-^{3}H and the efflux of radioactivity from the sacks was monitored (open circles). The broken line near the top of the figure represents the activity of *cis*-PPC-^{3}H in the initial dialysis bath.

at a fixed concentration of 3×10^{-5} M and the concentration of NaCl was varied. Figure 7 shows that the binding of platinum to DNA was severely impeded by chloride, indicating that loss of this ion is a prerequisite for binding. Noteworthy in this regard is the fact that binding was completely inhibited at a chloride concentration lower than that in the tissue culture medium or in plasma. Indeed, Fig. 8 shows that radioactivity which associated with DNA in the presence of 0·9% NaCl was present only as a result of passive diffusion, was not concentrated to a level greater than that outside the dialysis sack, and was totally removed after the sacks were transferred to a solution of 0·9% NaCl devoid of *cis*-PPC-^{3}H.

To assess any possible selectivity of binding to a single purine or pyrimidine, homopolymers of adenylic acid, cytidylic acid, guanylic acid and uridylic acid were dialyzed against *cis*-PPC-^{3}H. Solutions of the polynucleotides were equimolar with respect to the component mononucleotide. The results, shown in Fig. 9, indicate a substantially greater affinity of *cis*-PPC-^{3}H for polyguanylic acid and polyuridylic acid than for the other two homopolynucleotides. Further dialysis of the platinum–homopolymer complexes against distilled water or 0·9% NaCl resulted in removal of a substantial amount of radioactivity from the platinum-polyA and platinum-polyC solutions, but considerably less from the platinum-polyG and platinum-polyU solutions (Fig. 10).

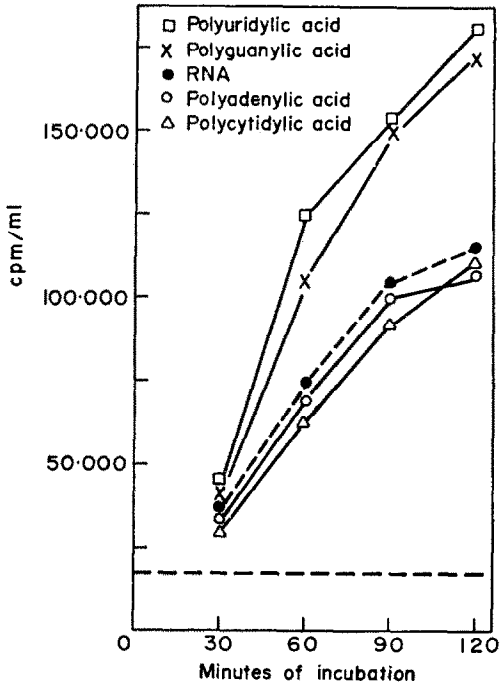

FIG. 9. Accumulation of *cis*-PPC-^{3}H across a semipermeable membrane into solutions of selected homopolynucleotides as compared with the accumulation into a solution of high molecular weight yeast RNA as a function of time. Solutions of the homopolymers were equimolar with respect to the component nucleotide; polycytidylic acid, containing the pyrimidine with the lowest molecular weight, was used at 0·05% (w/v). Yeast RNA was at 0·052 per cent, a value derived using the average molecular weight of the four component bases. The broken line represents the activity of *cis*-PPC-^{3}H (3×10^{-5} M) in the dialysis bath.

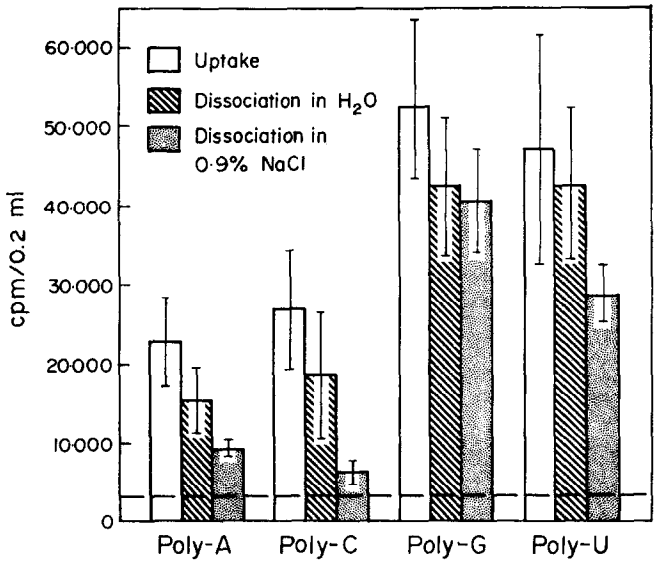

FIG. 10. Uptake of *cis*-PPC-^{3}H by selected homopolymers and its subsequent dissociation in water or 0·9% NaCl. Sacks containing the polymers were placed into a solution of *cis*-PPC-^{3}H (3×10^{-5} M) for 2 hr. After this period, sacks were removed and placed into either water or 0·9% NaCl for 2·5 hr. Data are a composite of three similar experiments. The broken line near the bottom of the figure represents the activity of *cis*-PPC-^{3}H in the initial dialysis bath.

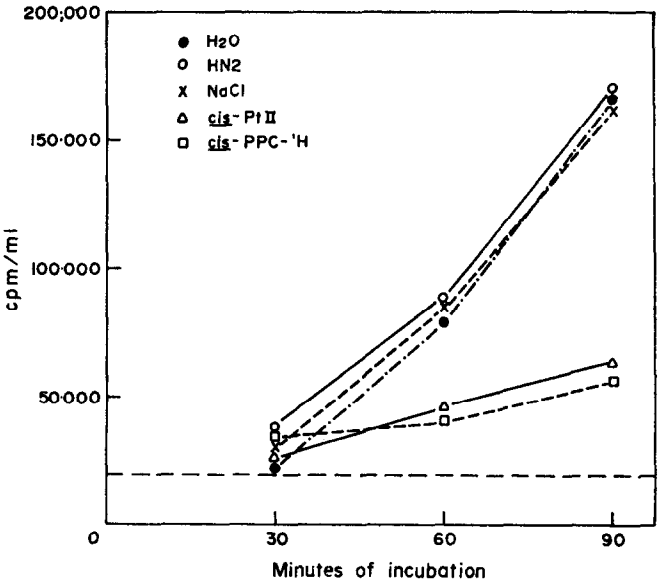

FIG. 11. Effects of dialyzing DNA against various compounds prior to dialysis in *cis*-PPC-^{3}H. Solutions of DNA were dialyzed in distilled water (closed circles), HN_2 ($1·5 \times 10^{-4}$ M; open circles) NaCl ($1·5 \times 10^{-4}$ M; crosses), *cis*-PtII ($1·5 \times 10^{-4}$ M; triangles) or *cis*-PPC-^{1}H ($1·5 \times 10^{-4}$ M; squares) for 3 hr; sacks were then transferred to distilled water for 30 min to remove any dialyzable material. The sacks were then placed into a bath containing *cis*-PPC-^{3}H (3×10^{-5} M) and samples were removed at intervals up to 90 min. The broken line near the bottom of the figure represents the activity of *cis*-PPC-^{3}H in the dialysis bath.

This binding of the platinum complex to nucleic acids is reminiscent of the action of the cytostatic alkylating agents. Indeed, using the dialysis system described, it was found that in distilled water HN_2-^{14}C ($1{\cdot}5 \times 10^{-4}$ M) accumulated in the DNA solution to a 10-fold extent after 1 hr. The presence of 0·005% NaCl totally prevented this accumulation during a 3-hr period of observation. To determine if HN_2 is competitive with *cis*-PPC-^{3}H, a DNA solution was dialyzed in a bath containing HN_2-^{12}C at $1{\cdot}5 \times 10^{-4}$ M for 3 hr. The sack containing alkylated DNA was then transferred to a bath containing *cis*-PPC-^{3}H in distilled water. Controls included sacks of DNA solution previously immersed in distilled water, 0·9% NaCl, *cis*-PPC-^{1}H, or *cis*-PtII for 3 hr. Figure 11 shows that the alkylation of DNA with HN_2 did not diminish at all the extent of subsequent platination of DNA with *cis*-PPC-^{3}H.

DISCUSSION

Cis-PPC-^{3}H forms an acid-resistant bond, possibly covalent, with intact cells and with purified DNA, but does not bind appreciably with protein, polysaccharide, or erythrocyte membranes. The platinum–nucleic acid bond is probably the result of electrophilic attack by the aquated platinum complex on some electron-rich portion of the nucleic acid molecule. It is of interest that chloride at a concentration of 0·1 per cent or less in the equilibrium dialysis system prevents any detectable binding to DNA (Fig. 7), whereas binding to an acid-insoluble fraction of viable tumor cells proceeds in spite of a chloride concentration of 0·8 per cent. The suggestion was made in an earlier report[2] that metabolic transformation of *cis*-PtII may be necessary for its inhibitory actions on tumor growth and DNA synthesis. In view of the present data on the binding of *cis*-PPC-^{3}H in cellular and non-cellular systems, enzymic participation in the former cannot yet be discounted.

The exact site of binding of the platinum complex to nucleic acid is not known; however, the present study suggests several possibilities. It is probable that *cis*-PPC-^{3}H does not bind to the sugar moiety of nucleic acid, since there was no appreciable accumulation of the complex into dextran solution during dialysis. It is quite probable that there is some interaction between the positively charged, aquated platinum complex and the negatively charged phosphate groups, but this would be labilized by increasing the ionic strength of the solution, as would result from addition of NaCl. The bonding of platinum to phosphate would not likely be resistant to repeated precipitation with TCA followed by dissolution in NaOH solution.

It is therefore proposed that the platinum forms a stable bond with purines and pyrimidines in the nucleic acid polymer at a site different from that bound by HN_2, and does so with some selectivity for guanine and uracil (thymine?). It is further proposed that the binding of platinum *in vivo* may well be facilitated by a catalyst (enzyme?), similar to the enzymatically mediated binding of HN_2 to sensitive cells.[9]

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