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STUDIES ON CIS-DICHLORODIAMMINEPLATINUM(II) AS A RADIOSENSITIZER

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Summary.—Cis-dichlorodiammineplatinum(II) (cisDDP) has been extensively studied as an antitumour agent; its binding to DNA has been proved but the radiosensitizing action has scarcely been tested. We report here that on TC.SV-40 mammalian cells cisDDP acts as a dose-modifying factor for ionizing radiation. The radiosensitizing action can be induced by two different mechanisms: reaction with non protein-SH groups and inhibition of repair processes. The cisDDP–DNA complex was studied against increasing radiation doses by analytical centrifugation and by spectrophotometrical measurements. The native complex seems to be more radioresistant than the denatured one.

AFTER the first interesting study of cis-dichlorodiammineplatinum(II) (cisDDP) as a cytotoxic agent, reported by Rosenberg (1969), the properties of this compound have been studied by several other authors (*e.g.* Connors *et al.*, 1972; Speer *et al.*, 1972, 1975) including its interaction with DNA (Howle and Gale, 1970; Horacek and Drobnik, 1971). However, we only found two reports (Zak and Drobnik, 1971; Wodinsky *et al.*, 1974) on the combined action of cisDDP with radiation. We have studied cis-dichlorodiammineplatinum(II) as a radiosensitizer, considering especially its physicochemical behaviour on DNA irradiated solutions, its effect on some molecular targets of radiation and its radiosensitizing action.

MATERIALS AND METHODS

Cell culture.—Radiosensitization *in vitro* was studied on TC.SV-40 cells, a mammalian cell line isolated from a golden hamster by Valladares and Alvarez (1969). Cells were grown in monolayer culture in R5, a MacCoy medium supplemented with 20% calf serum at 37°C. They were harvested by trypsinization from exponentially-growing cultures and suspended in GKN saline either to be treated with the drug or irradiated alone and with the product. Appropriate dilutions from the suspensions were plated on fresh medium, the dishes incubated at 37°C for 7–10 days and scored for colony formation.

Sulphydryl determination.—Non-protein-bound thiol (NPSH) was determined by the use of the Ellman reagent as described by Harris and Patt (1969). Approximately 5×10^6 TC.SV-40 cells harvested from exponential growth were exposed to 10^{-7} M cisDDP solution or kept in phosphate buffer pH 6·8 for increasing periods of 15, 30, 45 and 60 min at room temperature; the cells were then washed with the same buffer and NPSH extracted with 5% sulphosalicylic acid at 4°C in a homogeniser. The filtrants after Milipore filtration were assayed at pH 6·8 with 5,5′-dithiobis(2-nitrobenzoic acid).

DNA semiconservative and repair synthesis.—DNA synthesis was measured by ^{3}H-thymidine incorporation. Spleen mouse cells suspended in Hanks' medium, alone and with 10^{-2} M hydroxyurea were preincubated at 37°C for 1 h with and without 25 μM cisDDP. Then they were incubated for different times increasing up to 60 min at 37°C with 1·0 μCi/ml ^{3}H-methylthymidine and samples thoroughly washed with PCA 6%. DNA activity was measured from the acid-insoluble material after hydrolysis at 90°C with PCA in a liquid scintillation Mark II apparatus and the DNA concentration evaluated from its ribose content. Repair synthesis was induced in the cells by γ-irradiation with 30 krad before the isotopic labelling.

Molecular weight determination.—Sedimentation coefficients were determined in an

analytical ultracentrifuge (Beckman Instr.) with a scanning system (18,000 and 30,000 rev/min; absorption at 265 nm) on complexes and DNA samples from calf thymus at a concentration of about 20 μg/ml. Denaturation was performed by alkaline treatment. The complex was formed by addition of 10^{-3} M cisDDP to 400 μg/ml DNA solutions at room temperature. The integral distribution of the sedimentation coefficients was transformed into a mol. wt distribution by means of Eigner and Doty equations and, consequently, weight average mol. wt and number average mol. wt (Mw and Mn) were calculated.

Ultraviolet characterization of DNA-cisDDP interactions.—10^{-3} M cisDDP solutions were incubated at 37°C for 1 h with 40 mg/ml DNA saline solutions and γ-irradiated. Precipitates were isolated by centrifugation and hydrolysed at 90°C with PCA. Ultraviolet characterization in an Hitachi spectrophotometer were made on the first supernatant fraction and on the liquid from hydrolysis.

Irradiation.—The ^{60}Co γ-ray exposures were carried out in a pool source at a dose rate of 5·9 krad/min measured by Fricke dosimetry. Doses up to 675 krad were delivered. X-irradiations were performed in a 250 kV Isomatix equipment working at 180 kV 18 mA with 0·5 mm Cu and 1 mm Al filters at a dose rate of 57 rad/min for the cellular suspensions and at 250 kV 8 mA and 1 mm Cu with a dose rate of 200 rad/min for the DNA-cisDDP complexes.

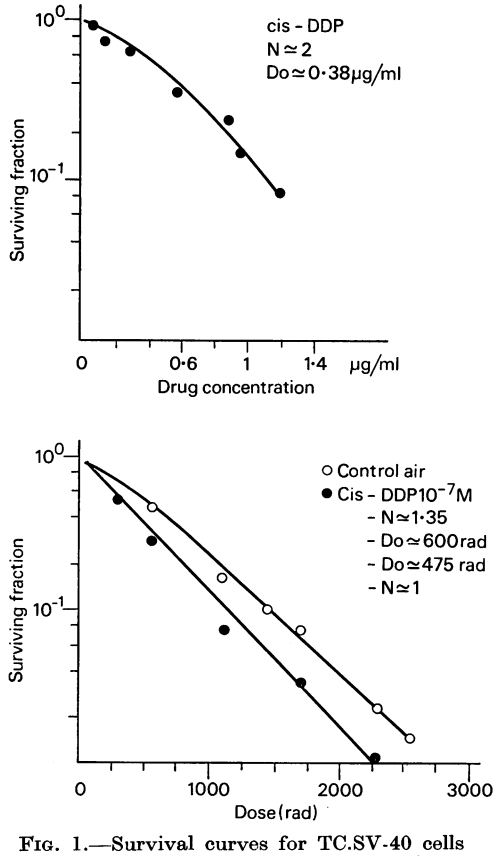

FIG. 1.—Survival curves for TC.SV-40 cells exposed to cisDDP and for cells irradiated in the presence of 10^{-3}M cisDDP.

RESULTS

Results collected in Fig. 1 include the toxicity survival curve studied with increasing cisDDP concentrations where a D_0 of 0·38 μg/ml was obtained, and the irradiation survival curves tested on control cells and on 0·03 μg/ml cisDDP-treated cells. The corresponding D_0 were 600 rad and 475 rad giving a dose-modifying factor of 1·26 in aerobic conditions.

Non-protein-SH levels at different incubation times with 10^{-7} M cisDDP are summarized in Table I. Each value represents the average of 3 or 4 determinations.

The effect of cisDDP on DNA semiconservative synthesis was shown to be dependent on the preincubation time; Fig. 2 presents the kinetics of two experiments differing only in this condition. While 10^{-2} M hydroxyurea decreases its inhibitory power, cisDDP increases its action on DNA semiconservative synthesis in more than a 100% inhibition with as long a preincubation time as 15 h. Repair synthesis results are represented in Fig. 3 and it can be seen that 25 μM cisDDP

TABLE I.

Incubation time (min)	Concentration — SH content μM/cell 10^{-9}	(%)
0	19·22 ± 0·75	100
15	11·46 ± 0·37	60
30	6·95 ± 0·30	36
45	5·60 ± 0·13	29
60	9·17 ± 0·71	47

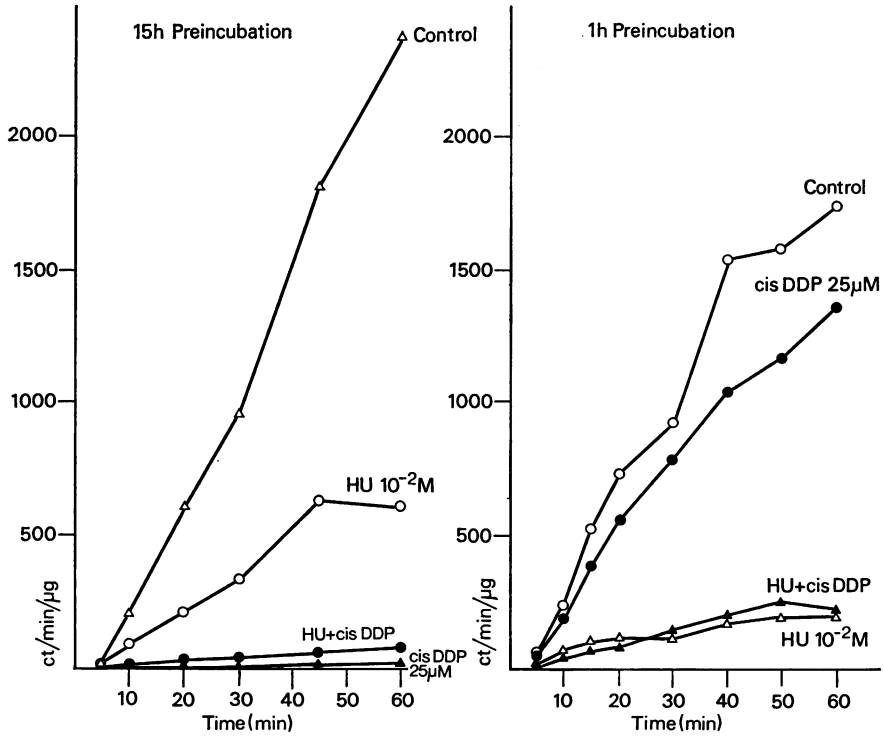

FIG. 2.—Effect of cisDDP on DNA semiconservative synthesis.

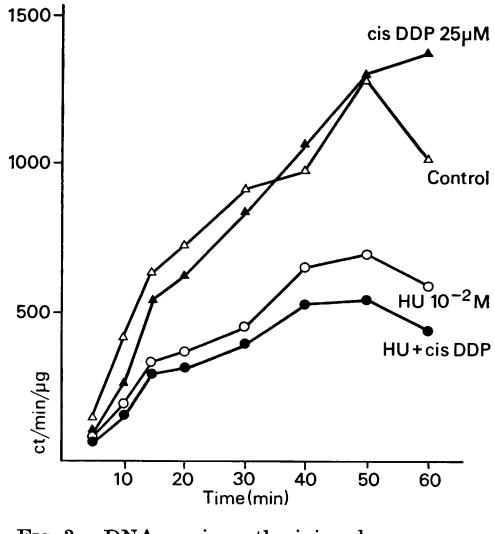

FIG. 3.—DNA repair synthesis in spleen mouse cells irradiated with 30 krad and exposed to cis-DDP with and without 10^{-2}M hydroxyurea.

increases by 15% the inhibitory action of hydroxyurea.

Ultracentrifuge data are collected in Table II, which shows an enormous increase in the sedimentation coefficients and in both weight average mol. wt and number average mol. wt from the cisDDP complex in relation to the DNA control values. Irradiation doses up to 18 krad seem to decrease the sedimentation coefficients, both in native and denatured complexes.

In Fig. 4 the ultraviolet spectra of two fractions obtained in the irradiation of cisDDP-DNA samples are depicted. While DNA remains in solution in the control sample, precipitation is induced by irradiation and the supernatants show no characteristics of DNA bases. The hydrolysed precipitates, on the contrary, present the absorption data characterizing

TABLE II.

Sample	Native			Denatured		
	S_{20w}	Mw 10^6	Mn 10^6	S_{20w}	Mw 10^6	Mn 10^6
DNA-cisDDP control	70·26	227·50	74·84	74·14	170·80	40·20
DNA-cisDDP 6 krad	67·68	199·30	73·74	57·08	73·79	23·46
DNA-cisDDP 12 krad	64·68	250	71·74	49·83	38·31	19·63
DNA-cisDDP 18 krad	62·26	155·9	62·19	43·81	41·86	13·85
DNA control	22·41	10·87	6·02	21·83	8·03	1·85

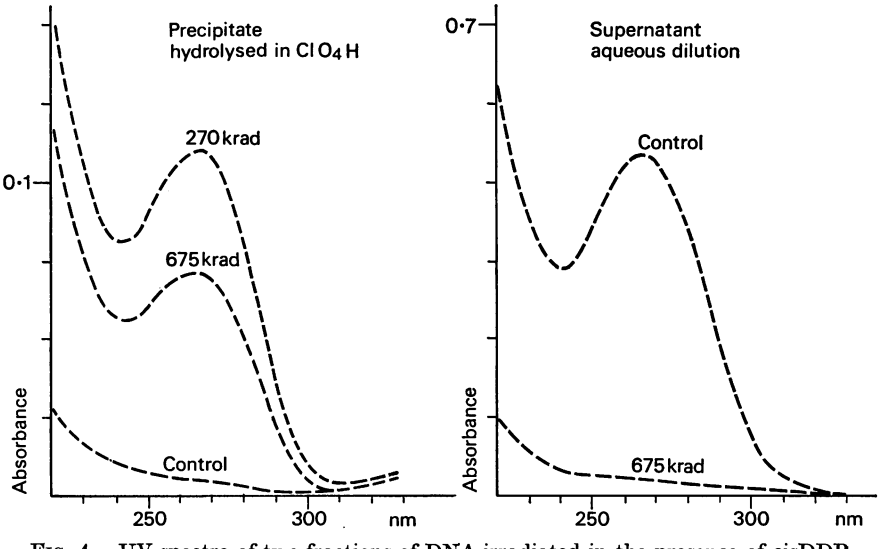

FIG. 4.—UV spectra of two fractions of DNA irradiated in the presence of cisDDP.

DNA with the normal degradation induced by high irradiation doses.

DISCUSSION

The survival curves from cellular radiosensitization *in vitro* show some clear differences. Besides the indicated D_0 values and the corresponding dose-modifying factor, sensitization is indicated by the disappearance of the shoulder in the survival curve of cisDDP-treated cells and by the decrease in the extrapolation number. Similar results are obtained with other radiosensitizers. The shoulder effect probably represents repair of the radiation damage and its disappearance would indicate that repair mechanisms have been completely inhibited. The control NPSH value is similar to that from Chinese hamster cells. The decrease with the incubation time and the later recovery was also exhibited by diamide and this shows that reversible removal of —SH groups occurs, once the reagent has been consumed in oxidative reactions, and that the treated cells quickly regenerate NPSH. This result would indicate equally that cisDDP may act as radiosensitizer by SH-scavenging mechanisms.

Our results on DNA semiconservative synthesis have reproduced the reported

dependence on the preincubation time with cisDDP. Nevertheless, the 15 h period is not a very good one for studying repair synthesis, cells being so damaged that the subsequent radiation effect disqualifies them for the DNA synthesis. A better incubation time is 2 or 6 h and in these conditions the radiosensitizing action is indicated by the inhibition of repair processes.

The physicochemical characterization of the interactions between DNA and cisDDP clearly show that a complex is formed: the maximum absorbence is shifted to longer wave-lengths and sedimentation coefficients increase both on native and denatured DNA. These latter data would be interpreted by the incorporation of cisDDP in both strands of DNA and denaturation is not prevented. Without forgetting that the equations for calculating Mw and Mn are usually applied on smaller sedimentation coefficients, we determined these values. The increase in percentage in the weight average mol. wts on native and denatured complexes are similar but the number average mol. wt in the denatured complex is larger than on the native one; this would indicate that cross-links are preferentially intrastrand. On the other hand, the complex is precipitated by high radiation doses; bases are degradated but always remain attached to platinum.

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