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NATURE OF TOXICITY FOR CHICK EMBRYO FIBROBLAST CELLS OF COUMERMYCIN A_1 AND ITS PHYSICO-CHEMICAL INTERACTIONS WITH PROTEIN AND NUCLEIC ACID

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Abstract—The results reported in this paper describe the effects produced by the antibiotic Coumermycin A_1 (CA_1) on survival and metabolism of chick embryo fibroblast cells (CEF), and give a clue to the understanding of its toxicity. The drug acts primarily at the level of DNA and RNA synthetic enzymes; no effect on DNA superstructure is detectable at doses at which cytotoxicity is pronounced. A spectroscopic approach produced evidence that CA_1 binds to DNA, RNA, chromatin components such as histones and to a structurally unrelated protein such as bovine serum albumin. Furthermore, CA_1 behaves like a pure non-competitive inhibitor of lactic dehydrogenase, a ubiquitous enzyme not involved in nucleic acid metabolism. The interaction of CA_1 with a wide range of macromolecules playing different biological roles is certainly relevant to its activity and adds a new insight into the mechanism of action of this antibiotic. These observations are also discussed in the light of the alleged role of CA_1 as a specific inhibitor of DNA topoisomerase in eukaryotic cells.

The use of inhibitors of DNA synthesis is sometimes a valuable approach for understanding the mechanisms of DNA replication and the roles of various enzymes, which are particularly complex in eukaryotic cells. Coumermycin A_1 (CA_1), a natural analogue of Novobiocin, is a coumarin- and carbohydrate-containing antibiotic produced by *Streptomyces richiriensis* [1, 2]. It is active against a variety of bacteria, particularly Gram-positive organisms [3]. It has also been shown to inhibit preferentially the replicative DNA synthesis in *E. coli* [4], in which system the drug is a specific inhibitor of the B subunit of DNA gyrase [5, 6]. For this reason it has also been employed in the search for topoisomerase II and gyrase-like activities in eukaryotes [7–9]. However, the biochemical evidence for the implication of these enzymes as specific targets of CA_1 in a DNA replication sensitive step does not seem to be conclusive.

Recently the antibiotic has also proved to be active in blocking the replication of DNA [10, 11] and RNA viruses [12], probably by affecting vital functions mediated by host cells, as suggested by the toxicity of the drug on BHK and CV_1 cells [10, 11].

The objective of the present work was to investigate the cytotoxicity of CA_1 for chick embryo fibroblast cells (CEF) by metabolic studies; in particular, experiments were performed to discover if any macromolecular synthesis was preferentially affected.

The reason for choosing CEF for our studies was dictated by their being natural hosts of different viruses and easily obtained in primary cultures, which, in contrast with cell lines, have not adapted their biosynthetic enzyme activities to conditions of continuous *in vitro* growth. Further attention was focused on the study of the DNA structure of treated cells, since recent reports on the parent compound Novobiocin were quite conflicting in defining the nature of changes occurring in DNA [13, 14].

In order to supplement the investigation on metabolism and cell survival, physico-chemical studies on the interaction of CA_1 with biological macromolecules involved in DNA and RNA synthesis were performed. Such studies were extended to other molecules selected as possible model targets of the drug.

MATERIALS AND METHODS

Chemicals and radioactive compounds. Coumermycin A_1 was a kind gift from Prof. R. E. Handschumacher (Yale University, Medical School). The antibiotic was dissolved in dimethyl sulphoxide (DMSO) and stored in small aliquots at −20°. DMSO (spectroscopic grade) and KI (extra pure) were purchased from Merck; ethidium bromide (EB), *N*-ethylmaleimide (NEM), phenyl methyl sulphonyl fluoride (PMSF) and dithiothreitol (DDT) were from Sigma. α-Amanitin was a kind gift from Prof. M. La Placa (University of Bologna, Medical School). Phosphocellulose (P-11) was purchased from Whatman and DEAE-Sephadex A-25 from Pharmacia.

Ribosomal RNA (Sigma) from rabbit liver was freed of contaminating protein and DNA by treatment with DNAse (Type I, Sigma) plus proteinase K (Type XI, Sigma), followed by several phenol extractions. Calf thymus DNA (Type I, Sigma) was

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activated as reported [15]; λ-DNA was obtained from Amersham. Histones (Type IIIs) and bovine serum albumin (BSA) fraction V were purchased from Sigma; LDH (from rabbit muscle) was from Boehringer Mannheim.

Unlabelled deoxynucleoside or ribonucleoside triphosphates were obtained from Boehringer, Mannheim. [^{3}H]methyl thymidine (specific activity 40–60 Ci/mmole), [5-^{3}H]uridine (specific activity 20–30 Ci/mmole) and L-[4,5-^{3}H]leucine (specific activity 100–150 Ci/mmole) were from the Radiochemical Centre (Amersham, Bucks., U.K.). [^{3}H]dTTP (30 Ci/mmole) and [5-^{3}H]UTP (23 Ci/mmole) were also from the Radiochemical Centre.

Cells and culture conditions. Primary cultures of CEF were obtained from 10-day-old embryonated eggs as described [16].

Cells were seeded into 3 and 6 cm diameter Petri dishes (Falcon) and cultured in Dulbecco modified minimal essential medium (DMEM) with the addition of 10% heat inactivated fetal calf serum (FCS, Gibco), HEPES (20 mM), antibiotics and L-glutamine (2 mM). Culture vessels were maintained in a moist atmosphere of 5% CO_2 in air.

Measurement of cell numbers, DNA, RNA and protein synthesis. Cells in a steady and exponential state of growth were incubated with various concentrations of CA_1 freshly made up in DMEM each time before use. Cell numbers were measured at the beginning and after the period of treatment by counting trypsinized cells in a conventional haemocytometer under phase contrast.

Pulse labelling experiments in the presence of the compound were carried out by adding to the culture for a period of 30 min 5 μCi/ml of the following metabolites: [^{3}H]thymidine, [^{3}H]uridine and [^{3}H]leucine, the latter in the medium without leucine. In some experiments the drug was removed by washing the cells extensively before incubation with the radioisotopes. At the end of the incubation period the cells were processed to measure TCA-precipitable radioactivity as described elsewhere [17].

Neutral sucrose density gradient analysis. The sedimentation properties of CEF nucleoids were examined by using the method of Cook and Brazell [18]. Cells were labelled by overnight incubation at 37° with 5 μCi/ml [^{3}H]thymidine and then incubated for 2 hr with fresh medium containing CA_1 but not radionuclides. In some experiments CA_1 was also added during the labelling period. Cells (5×10^5) in 50 μl of PBS were eventually added to 150 μl of lysis solution [18] and the mixture was layered on top of a preformed 15–30% sucrose gradient after 20 min at room temperature. In separate experiments different concentrations of EB were included in the sucrose solutions. Gradients were spun at 30,000 rpm for 30 min at 20° using a Beckman SW 50 rotor. Fractions were collected from the bottom of each tube and the radioactivity present was determined by liquid scintillation spectrometry as described in ref. [19].

Partial purification and assay of DNA polymerase α. CEF were used as the source of enzymatic activity. Cells obtained from 9 to 10 days fertilized eggs as described earlier were grown to confluence in large plastic flasks (Falcon). At this stage CEF were removed from the plates with a rubber policeman, harvested by centrifugation, washed three times with citrate–saline and resuspended at 10^8 cells/ml in lysis buffer [20] (50 mM Tris–HCl, pH 7.6, 0.1 mM K-EDTA, 0.2 mM DDT, 1 mM PMSF, 10% glycerol, 0.5 M KCl and Triton X-100 0.5%).

Cells were lysed by three cycles of freezing and thawing in liquid nitrogen and finally disrupted with three 10 sec bursts at 1 min intervals by using a microprobe of a Branson B12 sonifier set at a power of 20. The homogenate was centrifuged for 1 hr at 40,000 rpm in a Beckman SW 50.1 rotor. The supernatant was collected and partial purification of DNA polymerase α was achieved by ion-exchange chromatography first on a 15 ml DEAE-Sephadex A-25 column, then on a 5 ml phosphocellulose (P-11) column as described elsewhere [20, 21]. All operations were carried out at 4°. The assay conditions for DNA polymerases α were those described by Yamaguchi *et al.* [20] using a volume of 100 μl.

Partial purification and assay of RNA polymerase II. Cells were harvested and washed as described above and cell extracts were prepared for chromatography as reported by Lobban and Simonovitch [22]. The chromatographic separation of the enzyme through a DEAE-Sephadex A-25 column and the assay conditions were also performed according to these authors [22].

Spectrometric studies. The concentrations of CA_1 in DMSO and Novobicin in H_2O were determined spectrophotometrically using $E_{280} = 6 \times 10^4$ (M/cm) and $E_{307} = 600$ (M/cm), respectively. NADH concentration was also determined spectrophotometrically using an extinction coefficient of 6.22×10^3 (M/cm). For calf thymus DNA, λ-DNA and ribosomal RNA, E_{260} was taken as 6600 (M/cm). The measurements were carried out using solutions of nucleic acid prepared so as to have optical density units of 2, 1 and 0.4 at 260 nm, depending on the experimental requirements. No buffer was used, except where indicated.

In a typical experiment, 10 μl of a concentrated solution of CA_1 (1.53 mM) in DMSO was diluted to the desired concentration in doubly-distilled water, directly into the absorption or fluorescence cuvette.

The absorption spectra were recorded on a Perkin–Elmer 576 spectrophotometer having, when appropriate, the protein or nucleic acid control samples in the reference cell. In the visible range, this arrangement cancelled out the effect of light scattering due to the macromolecular phase and any slight absorption of these materials in the range 300–400 nm; moreover, in the UV range it enabled the contribution of the drug to the absorption of the mixture to be recorded separately. The optical density in the absorption maximum for cell, solvent and sample was no more than 1.0. The absorbance at 280 nm (where the measurements were performed) obeyed Beer's law up to concentrations of 28 μM.

Fluorescence measurements were performed by means of a Perkin–Elmer MPF 44A spectrophotofluorometer equipped with a DCSU2 unit. Standard conditions were fluorescence excitation at 347 nm with a 15 nm slit, and fluorescence emission at 447 nm using a 15 nm slit.

The temperature was kept constant at 25 ± 0.2° by means of a thermocryostat Braun 1441–1425. Commercial quartz cells (Hellma) with a 1 cm path length were used in all the experiments. Many chemicals are strongly fluorescent or are fluorescence quenchers, therefore glassware was washed with a mixture of ethanol and nitric acid before use.

The experiments performed to determine K_{app}, the apparent binding constant of CA_1 to either BSA or histones or DNA or RNA, were carried out keeping the antibiotic concentration constant; depending on the final concentration of the macromolecule, different ratios of nucleic acid or protein (P) to drug (D) were obtained. In order to achieve a better evaluation of the K_{app} at low P/D ratios, the equation of Papaphilis and Shaw [23] was used:

$$(C_P^0 \times C_L^0)/(A^0 - A) = [K_{app}(E^0 - E_c)]^{-1} + (E^0 - E_c)^{-1}[(C_P^0 + C_L^0) - (A^0 - A)/(E^0 - E_c)]$$

where C_P^0 and C_L^0 are the concentrations of protein or nucleic acid and of the drug, respectively, A and A^0 the absorbance of the ligand in the presence and absence of the bound drug, and E^0 and E_c the molar extinction coefficients of the free and bound ligand, respectively.

Fluorescence quenching results were plotted according to the Stern–Volmer equation [24], which can be written as:

$$F_0/F' = 1 + K(Q), \text{ with } K = K_q\tau$$

where F_0 and F are the fluorescence quantum yields in the absence and presence of the quencher, (Q) is the quencher concentration, K the Stern–Volmer constant, τ the fluorescence decay time in the absence of quencher, and K_q the quenching rate constant.

Experimentally, small aliquots (50 μl) of a concentrated KI solution (2 M) were added to a 2 ml sample solution with an absorbance of 0.1 at the excitation wavelength of 347 nm. Corrections for dilution were made when necessary; since KI does not absorb at the excitation wavelength, no correction for inner filter effects was required.

RESULTS

Cytotoxicity of CA_1

The addition of CA_1 to confluent monolayers of CEF showed that the drug was toxic for the cells in a steady-state of growth and that the celll survival was affected by the drug in a dose-dependent manner. Treatment with 20 μg/ml CA_1 for 24 hr produced a significant decrease in cell number (Table 1A). With such a drug concentration, cell viability began to decrease as early as 10–12 hr following addition of the compound to the culture dishes; eventually the cells lysed.

Lower doses of 5 and 10 μg/ml had only a minor effect on cell survival (Table 1A) and a concentration of 1 μg/ml was not toxic under such experimental conditions. The cytotoxicity of the drug was more pronounced when cell survival was studied on a population of CEF in an exponential phase of growth as shown in Table 1B. In this case the TD_{50} was found to be between 1 and 2 μg/ml.

Table 1. (A) Cytotoxicity of CA_1 for CEF in a stationary condition of growth

CA_1 (μg/ml)	Cell numbers at 24 hours ($\times 10^{-6}$)	% of control (taken as 100%)
0	2.3	100
1	2.3	100
5	2.1	94
10	1.8	84
20	1.1	48

To confluent monolayers of CEF (2.5×10^6 cells/dish) different drug concentrations were added. Cells were counted after an incubation period of 24 hr with the drug (see Materials and Methods).

Table 1. (B) Cytotoxicity of CA_1 for CEF in an exponential condition of growth

CA_1 (μg/ml)	Cell numbers at 72 hours ($\times 10^{-4}$)	% of control (taken as 100%)
0	24.6	100
0.5	19.5	79.2
1	16.4	66.6
2	6.3	25.7
5	1.1	4.5
10	0.8	3.2
20	0.3	1.3

To 3.625×10^4 adherent CEF, different drug concentrations were added. Cells were counted after an incubation period of 72 hr with the drug (see Materials and Methods).

Effect of CA_1 on nucleic acid and protein synthesis

Both DNA and RNA synthesis were affected within 90 min by 20 μg/ml CA_1, and after 6 hr the uptake of both uridine and thymidine was reduced to *ca* 10% of the initial value. An effect on protein synthesis, as determined by the incorporation of leucine, required a longer time and higher concentration of CA_1 (Fig. 1).

In some experiments the compound had also been removed from the culture by extensive washing, and then the cells were pulse-labelled for 30 min with [^{3}H]thymidine at various times in order to see if the blocking of DNA synthesis was reversible.

An exposure of 7 hr prior to pulse-labelling of the cells was enough to keep a constant level of inhibition over the following period of 12 hr (see Table 2).

Sedimentation of nucleoids from CEF treated with CA_1 in neutral sucrose gradients

A structure resembling nuclei (nucleoids), but depleted of protein, containing intact supercoiled and circular DNA [25] is obtained by lysing eukaryotic cells in a non-ionic detergent at high salt concentrations [18].

A conformational modification of DNA contained in nucleoids, caused by a variation in the degree of supercoiling, is reflected on sucrose gradients [18]. The sedimentation pattern of nucleoids from cells treated with two different concentrations of CA_1 was

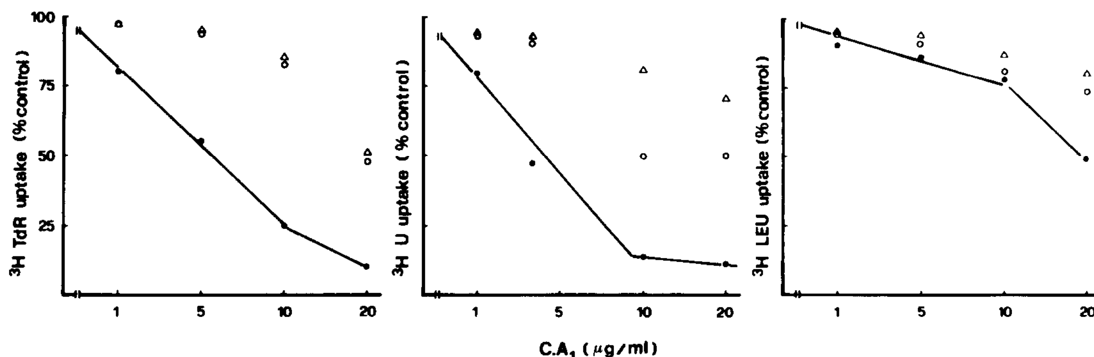

Fig. 1. Measurements of the incorporation of radiolabelled precursors of DNA, RNA and protein in CEF in pulse-labelling experiments at various concentrations of CA_1. ●, 6–6.5 hr; ○, 3–3.5 hr; △, 1–1.5 hr.

unchanged (see Fig. 2A). The same results were obtained independently when the cells were incubated with the drug during the entire pulse period or only during the chase (data not shown).

Titration of the degree of supercoiling in the DNA from nucleoids of treated and untreated cells was also performed by analysing the sedimentation profile of the two preparations in sucrose gradients, containing increasing amounts of ethidium bromide [26]. Low concentrations of the dye in fact induce the relaxation of supercoiled DNA; on the contrary, at higher dye concentrations, positive supercoiling occurs [27, 28]. These transitions are accompanied respectively by a drop and an increase in sedimentation rate, and a typical biphasic pattern is shown in Fig. 2B for CA_1-treated and untreated cells. In both, relaxation was more evident with a dose of 2 μg/ml ethidium bromide.

These data indicate that the extent of negative supercoiling is unaffected by CA_1 at concentrations at which the drug is cytotoxic and rapidly inhibits DNA synthesis.

Table 2. Residual activity of CEF ([^{3}H]Tdr incorporation) after exposure to CA_1 (20 μg/ml) for 7 hr: effect of drug removal

Hours after removal of CA_1	% residual activity*
2	80
4	80
12	85

* Values normalized to the activity present after treatment of 7 hr with CA_1, taken as 100%.

Assay of DNA polymerase α and RNA polymerase II

These enzymes were chosen in consideration of the metabolic studies which indicated interference of CA_1 with nucleic acid synthesis. *In vitro* assays showed that CA_1 inhibited DNA and RNA polymerases in a dose-dependent manner (Figs. 3A and 3B). It can be observed that RNA polymerase II,

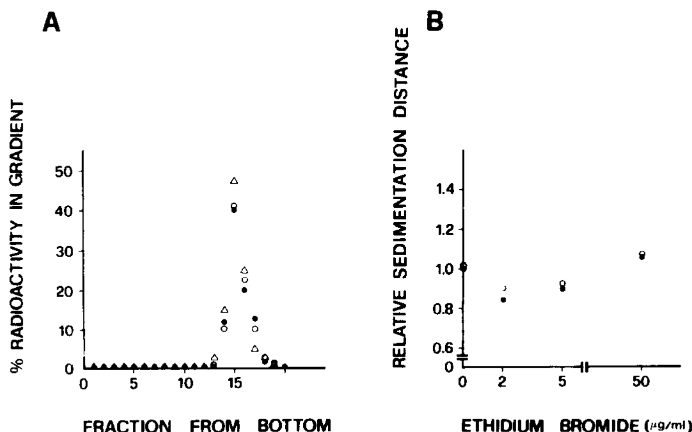

Fig. 2. (A) Sedimentation profile in a neutral sucrose density gradient of nucleoids from CEF. ●, Control. △, in the presence of 5 μg/ml CA_1; ○, in the presence of 20 μg/ml CA_1. (B) Sedimentation profile in a neutral sucrose density gradient of nucleoids from CEF in the presence of increasing amounts of ethidium bromide. The data are plotted as the ratio of sedimentation distances of the DNA from control nucleoids (●) and the DNA from treated cells (○). Each point represents the mean of three separate measurements.

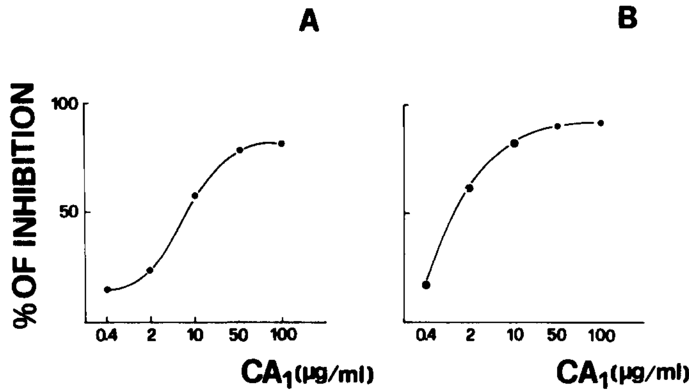

Fig. 3. Effect of CA_1 on DNA polymerase α (A) and on RNA polymerase II (B). (A) The reaction mixture of the α assay contained the following in 100 μl volume: 5 μmole Tris–HCl (pH 7.4), 100 nmole DTT, 800 nmole $MgCl_2$, 10 nmole each of dATP, dCTP and dGTP, 8 μg activated calf thymus DNA, 66 pmole [^{3}H]dTTP (30 Ci/mmole), 15% glycerol, 4 μg BSA, 3 μmole KCl and enzyme. Incorporation was normalized to the volume of control reaction lacking drug. Each point is the average of three determinations; 100% represents 0.330 pmole dTTP incorporated in 30 min at 37°. The reaction was shown to be sensitive to NEM [21]. (B) The reaction mixture for RNA polymerase II contained the following in 100 μl volume: 4.99 μmole Tris–HCl (pH 7.9), 0.166 mole $MnCl_2$, 0.62 mole NaI, 62.25 nmole each of ATP, CTP and GTP, 357 pmole [^{3}H]UTP (28 Ci/mmole), 0.25 μmole β-mercaptoethanol, 11.62 μg calf thymus DNA and enzyme. Incorporation was normalized to the volume of control reaction lacking drug. Each point is the average of three determinations; 100% represents 2.22 pmole UMP incorporated in 30 min at 37°. The specificity of the reaction is indicated by its intensity to α-amanitin [37].

under the experimental conditions employed, is slightly more sensitive to the drug than DNA polymerase α.

Interaction of CA_1 with LDH

In order to discover if the only target of the action of CA_1 were enzymes involved in DNA and RNA synthesis, we decided to study the possible interaction of the drug with an enzyme catalysing a reaction metabolically wide apart and ubiquitous such as LDH.

It is apparent from Figs. 4 and 5 that CA_1 interacts with the enzyme; it appears to act as a pure, non-competitive inhibitor with a K_m of 270 μM and a K_i of 5.7 μM.

Interaction of CA_1 with nucleic acid and protein: spectrometric studies

The results of the biological and enzymatic assays presented above prompted us to investigate if and

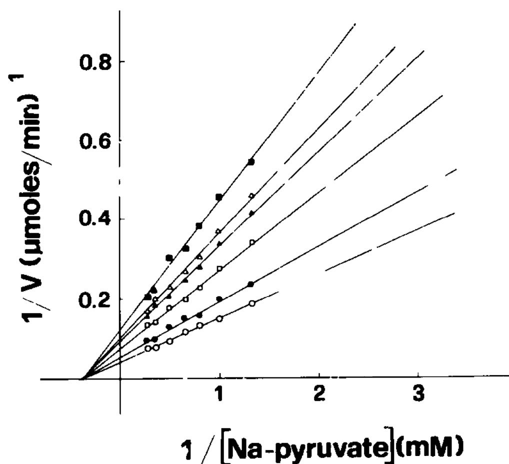

Fig. 4. Lineweaver–Burk plot of LDH activity in the absence (○) and presence of CA_1 (●, 3.6 μM; □, 7.9 μM; ▲, 11.5 μM; △, 14.4 μM; ■, 25.1 μM). The reaction mixture contained, in a final volume of 2 ml, 0.0011 mM LDH, 0.177 mM NADH and sodium pyruvate at increasing concentrations ranging from 0.05 to 0.275 mM, in 5 mM phosphate buffer. Initial rates of NADH oxidation, as deduced from the slope of velocity-progress curves [38], were used in all calculations.

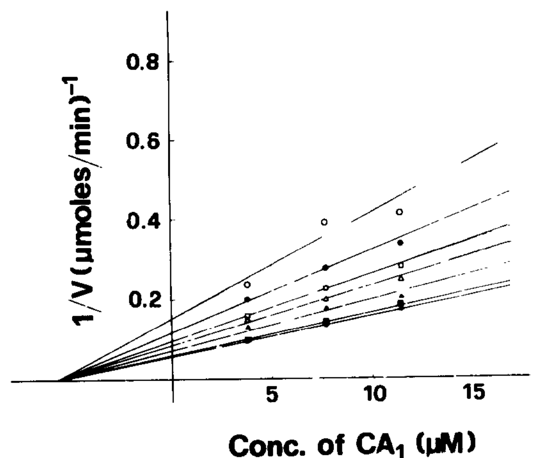

Fig. 5. Dixon plot of the inhibition of LDH activity by CA_1 (3.6, 7.9 and 11.5 μM) for different sodium pyruvate concentrations (○, 0.05 mM; ●, 0.075 mM; □, 0.10 mM; △, 0.125 mM; ▲, 0.150 mM; ■, 0.20 mM; ★, 0.275 mM).

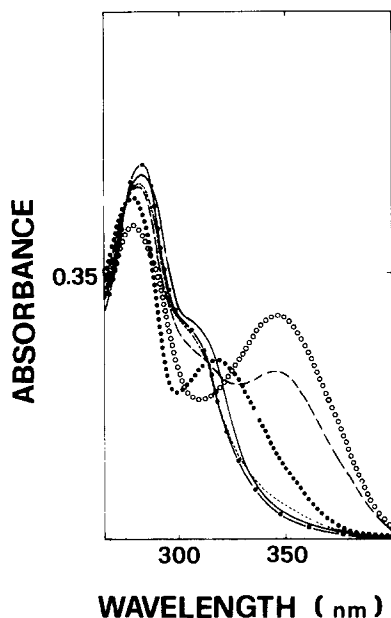

Fig. 6. Absorption spectra of CA_1 (7.5 μM) in different solvents and interacting with different macromolecules. OOO, 99.5% H_2O–0.5% DMSO; ——, 60 μM RNA; -●-, 60 γ/ml histones; ——, 60 γ/ml BSA; ---, 30 μM γ-DNA and calf thymus DNA; ●●●, DMSO.

Table 3. Affinity constants of CA_1 for different macromolecules

	P/D	K_{app}
CA_1-BSA	0.57–2.6	6.3×10^5
CA_1-Histones	0.48–2.9	8.7×10^5
CA_1-DNA	4.5–7.3	3.8×10^4
CA_1-RNA	4.9–10.9	2.6×10^4

how the drug interacts with other non-enzymatic proteins and with nucleic acids, which are natural substrates of some of the enzymes described.

The absorption spectra of CA_1 in an aqueous solvent (99.5% H_2O–0.5% DMSO), at a biologically significant concentration (7.5 μM), in DMSO and in the presence of BSA are shown in Fig. 6. The protein affected the absorption spectra appreciably: a hyperchromic effect of *ca* 14% together with a red shift of *ca* 8 nm for the band centred at 280 nm was observed. Also the band centred at 347 nm was blue-shifted *ca.* 30 nm. Similar but less-marked spectral modifications were produced in DMSO. The absorption spectrum of CA_1 in the presence of histones IIIs showed again a shoulder at 310 nm and the absorption maximum at 282 nm. The interaction of the drug with λ-DNA at a P/D ratio of one drug molecule/four base pairs caused the 347 nm band to disappear. On the contrary, such a band did not disappear in the presence of ribosomal RNA at the same, and higher, P/D ratios.

The fluorescence spectra, as shown in Fig. 7, followed a pattern similar to that of the absorption spectra. The emission band of CA_1 in 99.5% H_2O–0.5% DMSO resulted from the superimposition of four bands with the maxima centred at 429, 455 and 503 nm, respectively, as determined by the second derivative spectra (not shown). The fluorescence intensity was reduced above 3-fold in the presence of BSA and *ca* 2-fold in the presence of histones. Linear DNA and ribosomal RNA reduced the emission intensity about three times.

The apparent affinity constants of CA_1 with the proteins and nucleic acids tested are reported in Table 3. The values seem to indicate that the drug binds with higher affinity to the protein compared to the nucleic acids.

In order to investigate how deeply buried the drug becomes when interacting with BSA or DNA, fluorescence quenching experiments were performed with I^-. From an inspection of Fig. 8A, it is evident

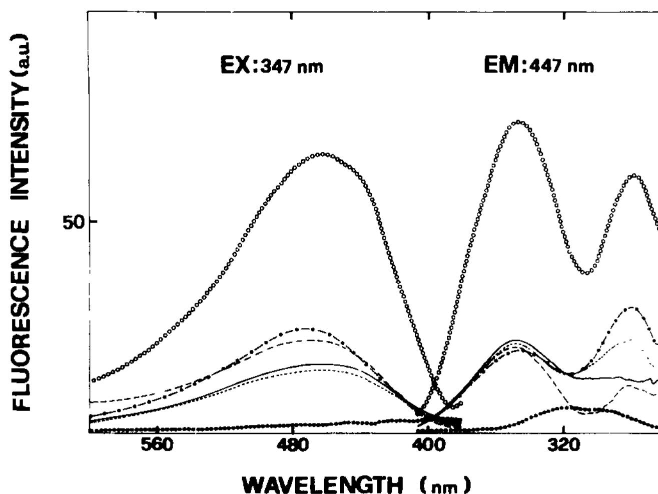

Fig. 7. Excitation and emission fluorescence spectra of CA_1 (1.65 μM) in different solvents and interacting with different macromolecules. OOO, 99.5% H_2O–0.5% DMSO; ——, 60 μM RNA; -●-, 60 γ/ml histones; ——, 60 γ/ml BSA; ---, 30 μM calf thymus DNA; ●●●, DMSO.

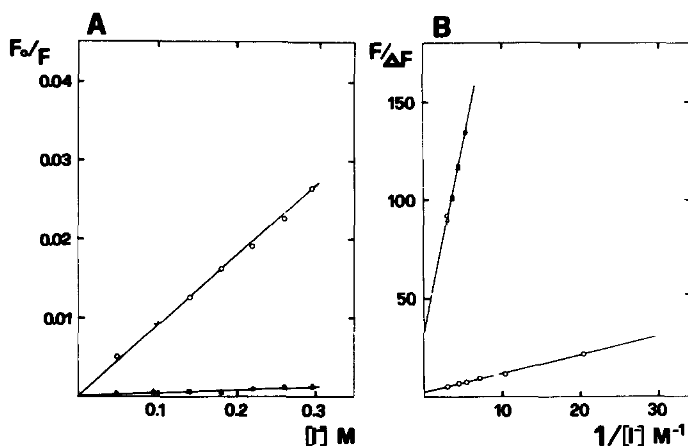

Fig. 8. (A) Stern–Volmer plots of the quenching of CA_1 fluorescence by I^- in water (○) and in the presence of BSA (△) and calf thymus DNA (□). (B) Inverse Stern–Volmer plots of the quenching of CA_1 fluorescence in water (○) and in the presence of BSA (△) and calf thymus DNA (□).

that CA_1 fluorescence is screened from the action of the quencher when the antibiotic interacts with the macromolecules. Figure 8B shows that only *ca* 40% of CA_1 fluorescence is indeed available to the action of the quencher.

DISCUSSION

The data presented here show that the antibiotic CA_1 is toxic for CEF in primary cultures and it is rapidly cytolytic at concentrations of 10–20 μg/ml. The cytotoxicity of the drug seems to stem from an action at the level of DNA and RNA synthetic enzymes, as documented by our metabolic studies with intact cells. Such studies in fact indicate that the interference with nucleic acid synthesis is an early biochemical lesion and that the effect on protein synthesis is probably secondary. The experiments on DNA polymerase α and RNA polymerase II, which are responsible, respectively, for the replication of chromosomal DNA [29, 30] and the synthesis of cytoplasmic messenger RNA [31] in superior cells, show an inhibition of these enzymes at doses at which the antibiotic is cytotoxic *in vivo*. These observations, while giving a clue to the pharmacological effect of the drug at the molecular level, do not support the assumption that CA_1 is active as an anti-topoisomerase in eukaryotic cells, as has been suggested in analogy to the prokaryotic model [9, 32]. Indeed, at doses at which cytotoxicity is pronounced, there is no detectable effect on DNA superstructure. Evidence for this is obtained by observing that nucleoids from treated and untreated cells have the same sedimentation rate in neutral sucrose density gradients and that their DNA has a comparable degree of negative supercoiling after ethidium bromide titration.

Identical types of studies carried out with Novobiocin in mammalian cells have produced conflicting results about changes occurring in nucleoid sedimentation, and no conclusive evidence has been derived for the mechanisms involved [13, 14]. Moreover, both CA_1 and Novovbiocin have been compared in order to study the effect on SW_{40} replication [10]. No alteration in supercoiling of the viral nucleic acid was observed following addition of CA_1 to the infected cells. On the other hand, the reduced degree of DNA supercoiling after treatment with Novobiocin [10] has been tentatively explained not by the involvement of a topoisomerase activity but as a consequence of inhibition of protein synthesis. More recent *in vitro* studies have shown that a topoisomerase from human fibroblasts [33] and *Xenopus laevis* [34] is sensitive to CA_1, but at extremely high doses. It appears, therefore, that for eukaryotic cells in general there is apparently no need to postulate an effect of DNA topoisomerase in order to explain the mechanism of the antibiotic activity.

In the course of this investigation, an additional interesting finding was the pattern followed by DNA synthesis after removal of the drug from cell cultures that had already shown signs of depressed metabolic activity at this level. Our data indicate that within the periods examined CA_1 inhibition of DNA synthesis is not reversible. This phenomenon could be explained by postulating the presence of acceptor molecules within the cell and the occurrence of a drug–acceptor complex that is quite stable once formed. In line with this thought, we decided to study the interaction of the drug with several macromolecules which may play this role.

We found that indeed CA_1 interacts with RNA and DNA and this is at variance with what has been previously reported. i.e. that while CA_1 interacts with BSA, no indication was found of its binding to DNA [35]. Furthermore, in addition to nucleic acids, CA_1 binds to histones IIIs leaving open the possibility that other chromatin components may interact with the drug, the interaction affecting their structure and activity. This would call for more complex mechanisms of action of the drug than those already proposed [3],

The physico-chemical and biochemical studies presented in this paper, however, indicate that CA_1 is not a selective inhibitor of topoisomerase in eukaryotic cells. It rather acts by affecting a wide

variety of macromolecules, some of them structurally (BSA) or enzymatically (LDH) unrelated to nucleic acid metabolism. The spectroscopic data indicate that the interactions of CA_1 with nucleic acid or protein should take place by means of the formation of weak bonds, as also shown to occur for furocoumarin derivatives [36].

It appears, therefore, that the toxicity of the antibiotic has to be ascribed to its aspecific interactions with several cellular components and not only with intermediates or enzymes of nucleic acid metabolism.

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