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# $^{32}$P-POST-LABELING ANALYSIS OF DNA ADDUCT FORMATION BY ANTITUMOR DRUG NITRACRINE (LEDAKRIN) AND OTHER NITROACRIDINES IN DIFFERENT BIOLOGICAL SYSTEMS

AGNIESZKA BARTOSZEK and JERZY KONOPA*
Department of Pharmaceutical Technology and Biochemistry, Technical University of Gdańsk, 80–952 Gdańsk, Poland

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**Abstract**—A $^{32}$P-post-labeling method has been employed to detect DNA adducts formed by derivatives of nitro-9-aminoacridine in both cellular and non-cellular systems. The treatment of HeLa $S_3$ cells in culture or Ehrlich ascites tumor cells *in vivo* with nitracrine and two other antitumor 1-nitro-9-aminoacridines, denoted C-857 and C-1006, resulted in covalent binding of these compounds to cellular DNA. Each derivative studied gave rise to a distinct pattern of adduct spots and the similarity of the respective adduct profiles was noted for the both cellular models. Calf thymus DNA samples modified *in vitro* with nitracrine and C-857 in the presence of either rat hepatic microsomal fraction or dithiothreitol yielded chromatographic profiles resembling those obtained in the cellular systems, suggesting similarity in the DNA adduct structures. There were also neither qualitative nor quantitative differences in calf thymus DNA modification by these two 1-nitro derivatives between aerobic and anaerobic conditions, thus the reduction of a nitro group seems not to be the only determinant of covalent binding to DNA *in vitro*. No DNA adduct formation was detected in the cellular systems used with 2-nitro and 4-nitro isomers of nitracrine that are devoid of cytotoxic activity, which provides further evidence that both covalent binding and DNA crosslinking, but not intercalation, are responsible for cytotoxic and antitumor properties of 1-nitro-9-aminoacridines.

A number of 1-nitro-9-amino derivatives of acridine, developed in this Department, exhibit potent cytotoxic and antitumor properties [1, 2]. One of these compounds, 1-nitro-9-(3′-dimethylamino-n-propylamino)acridine, has been used clinically in Poland under the name of Ledakrin (in this paper WHO recommended name nitracrine is used) for several years [3, 4]. Another one, denoted C-857, is in the process of being accepted for clinical studies.

The usefulness of 1-nitro-9-aminoacridines in cancer chemotherapy prompted the extensive investigations aimed to elucidate the mode of action of these compounds and to establish the relationship between their chemical structure and biological activity. Since acridine was known to intercalate into DNA, this phenomenon was initially thought to underlie the biological activity of 1-nitroacridines. A series of experiments demonstrated that several derivatives of nitro-9-aminoacridine possessing a nitro group in different positions of an acridine moiety were indeed potent DNA intercalators [5]. However, in cell systems in which 1-nitroacridines exhibited significant (e.g. higher than mitomycin C) cytotoxic activity, the derivatives with a nitro group in other positions of an acridine core proved to be inactive [6]. This finding raised doubts about the formerly postulated relevance of intercalation into DNA for biological activity of 1-nitroacridines. In addition, it turned out that nitracrine was a latent form of the drug and its cytotoxicity occurred only after metabolic activation [5, 7]. The activation, proceeding via both oxidative and reductive pathways, resulted in covalent binding of nitracrine to DNA and other cellular macromolecules [7, 8]. Closer examination of the nature of the former phenomenon revealed that nitracrine, as well as other 1-nitro-9-amino derivatives of acridine, was able to induce covalent interstrand crosslinks in DNA of mammalian and bacterial cells [6]. Furthermore, it was shown that there was a correlation between the crosslinking potency of 1-nitroacridines and their *in vivo* antitumor activity against Sarcoma 180 solid tumor bearing mice, as well as *in vitro* cytotoxicity against cultured HeLa $S_3$ cells [9]. These correlations indicated that covalent binding to DNA in general, and DNA crosslinking in particular, rather than intercalation into DNA, represented the crucial events responsible for antineoplastic properties of 1-nitro-9-aminoacridines.

The studies on DNA crosslinking by 1-nitro-9-aminoacridines allowed one to assume that covalent binding to DNA is a common feature of their mode of action, though it was directly confirmed only for nitracrine, and the deeper insight into DNA adduct

* To whom reprint requests should be sent.

† Abbreviations used: $EC_{50}$, compound concentration inhibiting by 50% cellular growth (cytotoxic activity); (PEI)-cellulose, polyethylene-imine-cellulose; BICINE, *N,N*-bis(2-hydroxyethyl)-glycine; DTT, dithiothreitol; SDS, sodium dodecyl sulphate; TCA, trichloroacetic acid; PBS, phosphate buffered saline (0.17 M NaCl, 0.27 mM KCl, 8.1 mM $Na_2HPO_4$, 0.2 mM $KH_2PO_4$, pH 7.2); TLC, thin layer chromatography; HPLC, high performance liquid chromatography; i.p., intraperitoneal.

formation by these derivatives became of immediate concern. Preliminary experiments, carried out with the aid of isotopically labeled nitracrine, led to the separation of five nitracrine-nucleoside adducts, as a result of the fractionation of the enzymatic digest of DNA isolated from Ehrlich ascites cells exposed *in vivo* to the drug [8]. Unfortunately, the requirement of radiolabeled derivatives restricted such studies to nitracrine.

In extension of these earlier experiments, a $^{32}P$-post-labeling assay [10, 11], an only recently developed method, which does not require isotopically labeled test chemicals, was applied to the analysis of DNA adducts formed by nitroacridines of diverse chemical structures. In this paper we describe results obtained by applying the above method to the detection of covalent DNA addition products of nitroacridines in different biological systems. We also present further data to support the hypothesis pointing out the importance of covalent binding to DNA, as opposed to DNA intercalation, for biological activity of 1-nitroacridines. Part of this work has been published in preliminary form [12].

## MATERIALS AND METHODS

*Materials.* Nitracrine, 1-nitro-9-(3′-dimethylamino-n-propylamino)acridine, and its analogues in the form of either mono- or dichlorides were kindly provided by the late Professor Andrzej Ledóchowski from this Department. [$^{14}C$]-nitracrine, specific activity 9 mCi/mmole, was synthesized at the Institute of Nuclear Research, Warsaw, Poland. The radiochemical purity of [$^{14}C$]-nitracrine was about 70% as determined by TLC with unlabeled nitracrine run as a standard and radioactivity scans of the chromatograms. HeLa $S_3$ cells, the media, and foetal calf serum were from Gibco-Biocult (Uxbridge, U.K.), Micrococcal endonuclease (Sigma Chemical Co., St Louis, MO) was dissolved in solution of spleen exonuclease (Boehringer, F.R.G.) dialysed against 40 mM sodium succinate, 20 mM $CaCl_2$, pH 6.0. T4 polynucleotide kinase (Pharmacia, Uppsala, Sweden), potato apyrase (Sigma Chemical Co., St Louis, MO) and proteinase K (Merck, Darmstadt, F.R.G.) were used without purification. Deoxyribonucleotides were obtained from Pharmacia (Uppsala, Sweden), (PEI)-cellulose thin layers from Merck (Darmstadt, F.R.G.), Hydroxylapatite (Bio-Gel HTP, DNA grade) from Bio-Rad Laboratories (Richmond, CA), nitrocellulose filters (type SM 113, 0.45 $\mu$M) from Sartorius GmbH (F.R.G.), calf thymus DNA, spermine, NADPH and BICINE from Sigma Chemical Co. (St Louis, MO), Nicotinamide from Loba (Austria), glucose-6-phosphate from Koch-Light (Colnbrook, U.K.) and DTT from Serva (Heidelberg, F.R.G.). $\gamma$-[$^{32}P$]-ATP was purchased either from Amersham International (Amersham, U.K.), (3000 Ci/mmole) or from Institute of Biochemistry and Biophysics, Polish Academy of Science, Warsaw, Poland, (800–1200 Ci/mmole). All other reagents used were analytical grade.

Microsomes were prepared as described previously [13] from livers of male Wistar albino rats weighing 180–240 g (obtained from Department of Pathology, Medical Academy, Gdańsk, Poland) and were frozen at −20° until used. The protein content of the microsomal preparations was determined by the modified method of Lowry [14].

*Treatment of tumor cells and DNA isolation.* HeLa $S_3$ cells were grown in spinner culture in Joklik's modified minimal essential medium supplemented with 5% foetal calf serum and antibiotics. The exponentially growing cells were treated with freshly prepared water solutions of nitroacridines as specified in the legends to the figures. Ehrlich ascites tumor cells were transplanted i.p. ($3 \times 10^6$ cells/mouse) into BALB/c female mice weighing about 20 g obtained from our breeding colony. The animals were maintained on a commercial diet and water *ad lib.* On the ninth day after transplantation the mice were administered i.p. a single dose of test nitroacridines in 0.25–0.5 ml of saline, the detailed treatment conditions are given in the legend to Fig. 1. Control animals received the solvent alone. The mice were killed by cervical dislocation, the ascites fluids were aspirated and diluted with ice cold PBS. DNA isolation was performed by the modified procedure of Rajewski *et al.* [15] which was adopted for cell suspensions and rendered less time-consuming. Briefly, the HeLa $S_3$ cells or Ehrlich ascites tumor cells were collected by centrifugation, washed twice with cold PBS and homogenized in Dounce homogenizer in 1% SDS. The homogenates were incubated with 250 $\mu$g/ml of proteinase K for 90 min at 38° and then extracted with chloroform:isoamyl alcohol (24:1). DNA was purified using hydroxylapatite (1 g/50 million cells) and extensively dialysed against distilled water ($3 \times 30$ min, 1 l/sample) by thin layer dialysis [16]. The amount of DNA was determined spectrophotometrically ($A_{260}$) and its purity was estimated from $A_{260}$:$A_{280}$ ratio. The DNA solutions were immediately submitted to the further experimental procedure, unless otherwise specified.

In vitro *modification of calf thymus DNA.* For *in vitro* modification, calf thymus DNA was incubated with 50 $\mu$M of nitracrine or C-857 in the presence of either rat hepatic microsomal fraction or DTT. The complete incubation mixture in the former system consisted of the drug, 0.5 mg/ml of calf thymus DNA, 0.5 mg/ml of microsomal protein, 1 mM NADPH, 15 mM glucose-6-phosphate, 12.5 mM nicotinamide, 10 mM $MgCl_2$ in phosphate buffer, pH 7.4. After 15 min of preincubation at 37° without substrate, the reaction was initiated by the addition of the drug and the mixture was further incubated at 37° for 1.5 hr. Where incubations were conducted under nitrogen, the incubation mix was purged with deoxygenated $N_2$ for about 20 min, then sealed for the duration of the incubation. Control mixtures included an appropriate amount of heat-inactivated microsomes (100°, 10 min) or buffer in place of the microsomes.

The modification of calf thymus DNA in the other non-cellular system was carried out essentially following the procedure described by Gniazdowski *et al.* [17]. The incubation mix comprised 50 $\mu$M of either 1-nitroacridine, 100 $\mu$g/ml of calf thymus DNA, 2 mM DTT, 0.1 M KCl in phosphate buffer, pH 7.4. The blanks were devoid of DTT. Incubation was conducted at 37° for 1 hr.

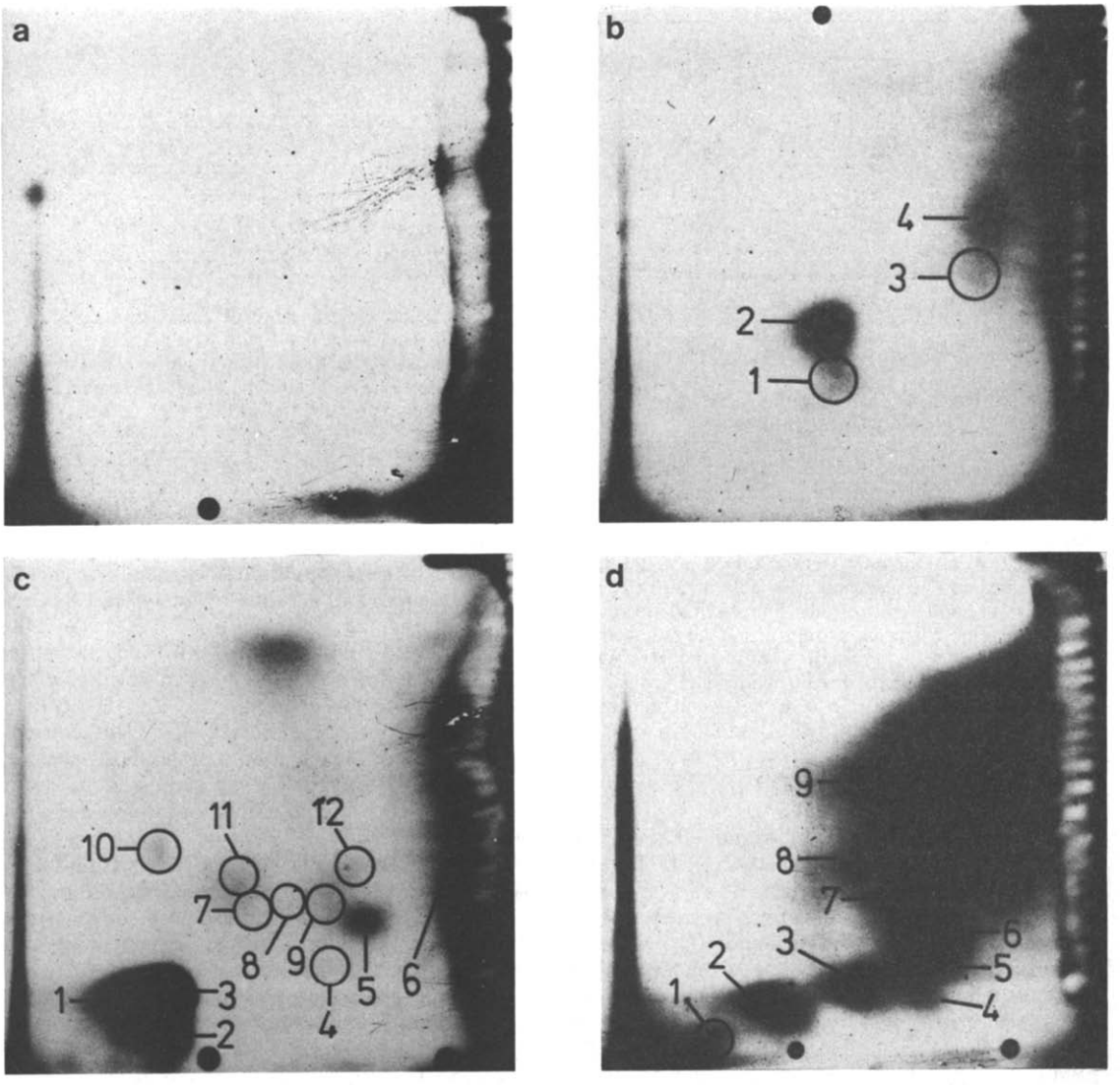



Fig. 1. Autoradiograms of DNA from Ehrlich ascites tumor cells treated *in vivo* with the derivatives of 1-nitro-9-aminoacridine, (a) control non-treated cells, (b) 0.3 mg/mouse nitracrine, (c) 0.2 mg/mouse C-857 and (d) 1.11 mg/mouse C-1006. The BALB/c mice were administered a single i.p. dose of the drugs for 4 hr. The animals were killed by cervical dislocation, the ascites fluids were aspirated, diluted with 0.9% NaCl solution and Ehrlich ascites cells were collected by centrifugation. The DNAs were isolated, digested to dNps, which were subjected to $^{32}$P-labeling and the individual adducts were resolved by TLC (see Materials and Methods for details). Approximately 120 $\mu$Ci was applied to the chromatograms. Spots were visualized by autoradiography at −20° for 7 days.

The *in vitro* reactions were terminated by the addition of an equal volume of chloroform:isoamyl alcohol (24:1). The aqueous layers were then submitted to the DNA isolation procedure as described above.

*DNA digestion and $^{32}$P-fingerprinting of DNA adducts*. Digestion and $^{32}$P-labeling conditions were similar to those described earlier [10, 11]. About 1 $\mu$g of DNA modified with derivatives of nitroacridine in either cellular or non-cellular systems was digested to nucleotides in 10 $\mu$l of 20 mM sodium succinate, 10 mM $CaCl_2$, pH 6.0, containing 6.25 $\mu$g of spleen exonuclease and 2.2 $\mu$g of micrococcal endonuclease, at 38° for 2 hr. The nucleotides were then converted to $^{32}$P-labeled deoxyribonucleotide-3′,5′-diphosphates. The reaction mix consisted of 2 $\mu$l of freshly prepared DNA digest, 4 $\mu$l of $\gamma$-[$^{32}$P]-ATP (150–180 $\mu$Ci), 2 $\mu$l of 150 mM ATP, 3 $\mu$l of 0.1 M BICINE–NaOH, 0.1 M $MgCl_2$, 0.1 M DTT, 10 mM spermine, pH 9.0 and 11.5 U of T4 polynucleotide kinase. The reaction mixtures were incubated for 60 min at 38°, then the excess ATP was destroyed by adding 4 $\mu$l of a freshly prepared 1:1 mixture of potato apyrase 7.5 mU/$\mu$l and solution of dpAp, dpGp, dpCp and dpTp (1.6 $\mu$g/$\mu$l each). The mixtures were kept at 38° for an additional 30 min. The individual adducts were resolved by 4-directional TLC [11], where the concentration of urea in D3 and

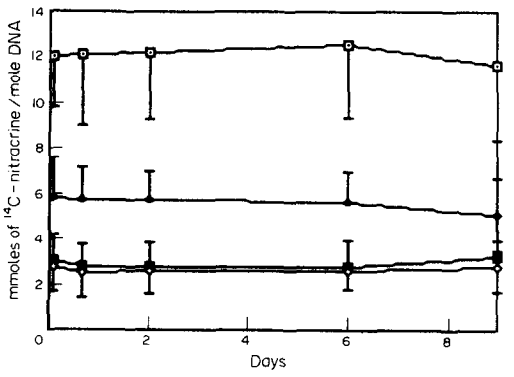


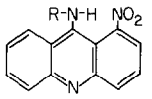

Fig. 2. Persistence of [$^{14}$C]-nitracrine covalent binding in isolated DNA. HeLa $S_3$ cells were treated with 2 μg/ml of [$^{14}$C]-nitracrine for 4 hr (—◆—), calf thymus DNA was incubated *in vitro* with 50 μM of [$^{14}$C]-nitracrine either in the presence of microsomes for 1.5 hr in aerobic (—■—) and anaerobic (—◇—) conditions or in the presence of 2 mM DTT for 1 hr (—⊡—). The DNAs were then isolated and stored at 4°. Aliquots were taken at the indicated times, DNA was precipitated with cold 10% TCA and radioactivity bound was assessed in liquid scintillation counter. Results were corrected by subtracting the radioactivity of control samples in which [$^{14}$C]-nitracrine was added directly to HeLa $S_3$ lysate or calf thymus DNA was incubated with 50 μM [$^{14}$C]-nitracrine with omission of, respectively, either microsomes or DTT. Data are expressed as means ± SE for three experiments.

D4 chromatographic solvents amounted to 7.5 M. Autoradiography was performed at −20° for 3–7 days after which the adduct spots and adjacent areas were excised and their radioactivity was determined in toluene-based scintillation fluid. The concentration of the adducts was estimated from the relative adduct labeling index as defined by Gupta *et al.* [10].

*Persistence of irreversible binding of* [$^{14}$C]*-nitracrine with DNA.* The conditions of HeLa $S_3$ cells treatment as well as that of calf thymus DNA modification *in vitro* were the same as in $^{32}$P-post-labeling assays, only isotopically labeled nitracrine (9 mCi/mmole) was used instead of the non-labeled compound. The [$^{14}$C]-nitracrine associated DNA was isolated as described above and stored at 4°. At the times indicated in the legend to Fig. 2, aliquots of DNA were precipitated with cold 7.5% TCA, collected on nitrocellulose filters and washed extensively with cold 5% TCA and ethanol. Radioactivity was determined in toluene-based scintillation fluid and mmoles of [$^{14}$C]-nitracrine bound to 1 mole of DNA calculated. Blank values for non-specific binding were obtained in the case of HeLa $S_3$ cells by adding [$^{14}$C]-nitracrine after proteinase K digestion to lysed non-treated cells, purifying the DNA from this lysate and determining the radioactivity associated with this preparation. In the case of non-cellular systems, for blank preparations calf thymus DNA was incubated with 50 μM [$^{14}$C]-nitracrine with omission of, respectively, either microsomes or DTT.

## RESULTS

*The analysis of DNA adduct formation by 1-nitro-9-aminoacridines with DNA of* HeLa $S_3$ *cells.* $^{32}$P-post-labeling analysis of DNA adduct formation was carried out for three derivatives of 1-nitro-9-aminoacridine, nitracrine, C-857 and C-1006, which differ in the structures of the amino side chain. The chemical formulae and $EC_{50}$ values against HeLa $S_3$ cells are shown in Fig. 3. The conditions of HeLa $S_3$ cells' treatment with nitracrine (2 μg/ml, 4 hr) were the same as those for which the maximal level of [$^{14}$C]-nitracrine covalent binding to DNA of HeLa cells had been previously observed [8]. The concentrations used for the remaining compounds, specified in the legend to Fig. 4, were adjusted so as to constitute the same multiplicity of $EC_{50}$ values as in the case of nitracrine (1360 × $EC_{50}$).




| COMPOUND | R | $EC_{50}$(nM) |
|---|---|---|
| LEDAKRIN | $-(CH_2)_3N(CH_3)_2$ | 3.7 |
| C-857 | $-(CH_2)_2OH$ | 0.5 |
| C-1006 | $-(CH_2)_3NH(CH_2)_5CH_3$ | 13.0 |

Fig. 3. Structures of the 1-nitro-9-aminoacridines studied used as hydrochlorides and their cytotoxic activities ($EC_{50}$) against HeLa $S_3$ cells. $EC_{50}$ values are drug concentrations inhibiting by 50% cell growth after 72 hr treatment as described elsewhere [9].

The isolation and digestion of DNAs from HeLa $S_3$ cells exposed to 1-nitroacridines, followed by $^{32}$P-labeling and fingerprinting procedures (see Materials and Methods) demonstrated that all the compounds studied gave rise to distinct patterns of chromatographic spots corresponding to DNA adducts. Representative autoradiograms, observed in four to eight independent assays, are given in Fig. 4. No adduct spots were detected in chromatograms from cells that had received solvent alone as controls (Fig. 4a).

The typical map of $^{32}$P-labeled digest of nitracrine-modified DNA from HeLa $S_3$ cells exhibited four spots, labeled as adducts one to four (Fig. 4b), that were not present in DNA digests of untreated cells. The major adduct, spot 2, represented 40% of the total adduct-associated radioactivity. The level of modification of DNA amounted to 1 adduct in about 485 nucleotides, as calculated from the total adduct radioactivity and the radioactivity of the normal nucleotides. This result is in accord with the data on DNA binding of [$^{14}$C]-nitracrine shown in Fig. 5. It also coincides with the previous estimations, obtained under similar conditions by a different procedure employing also [$^{14}$C]-nitracrine, where the extent of covalent binding to HeLa cells DNA was demonstrated as being 1 molecule of the drug per 370 DNA base pairs [8].

The autoradiograms from the C-857-modified DNA of HeLa $S_3$ cells (Fig. 4c) showed at least 12 distinct adduct spots clustered near the centre of the chromatogram. The most intensive were the adducts of low $R_F$ values, spot 1 and 3, which constituted about 41% of the total. Under the conditions

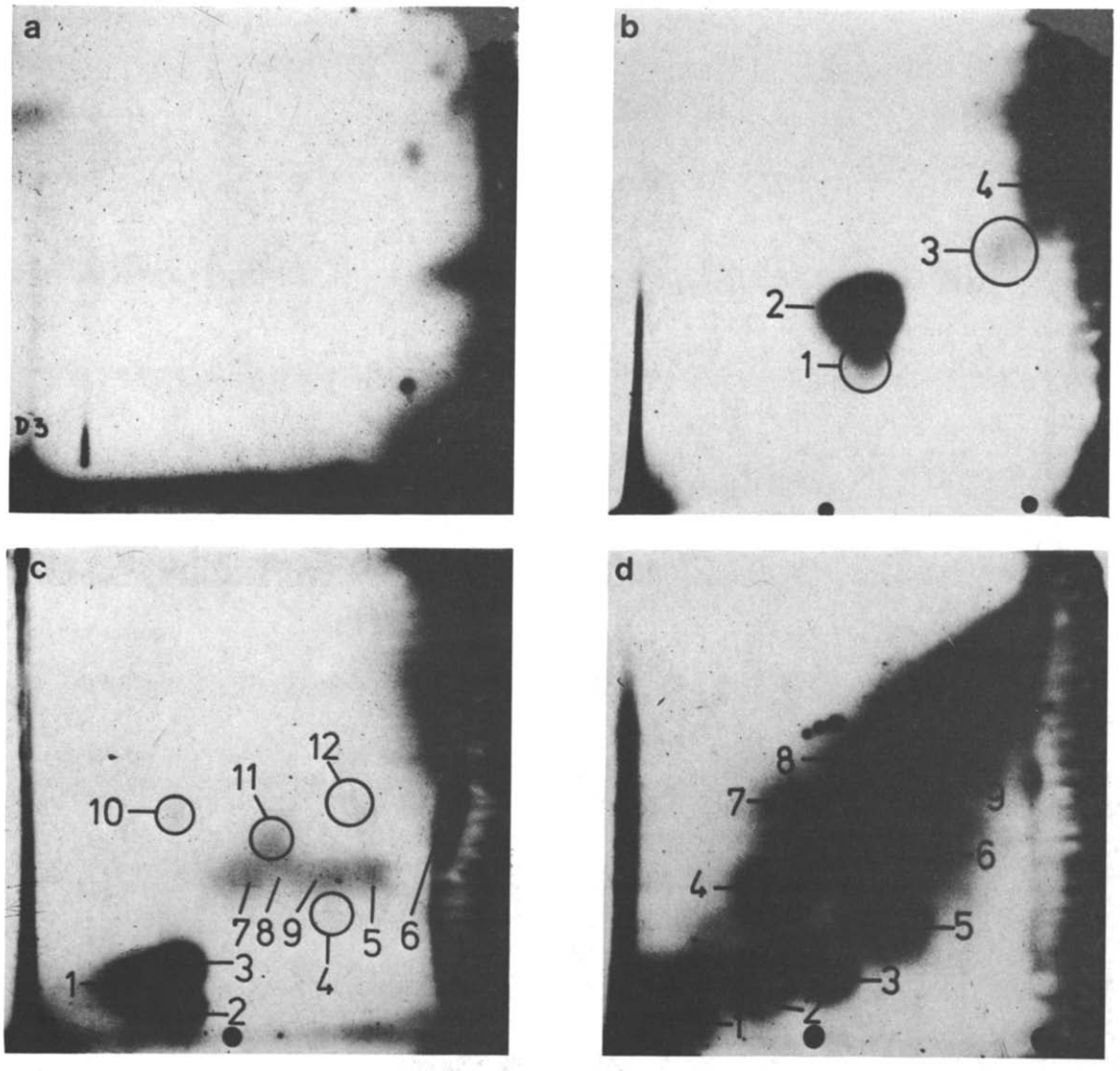
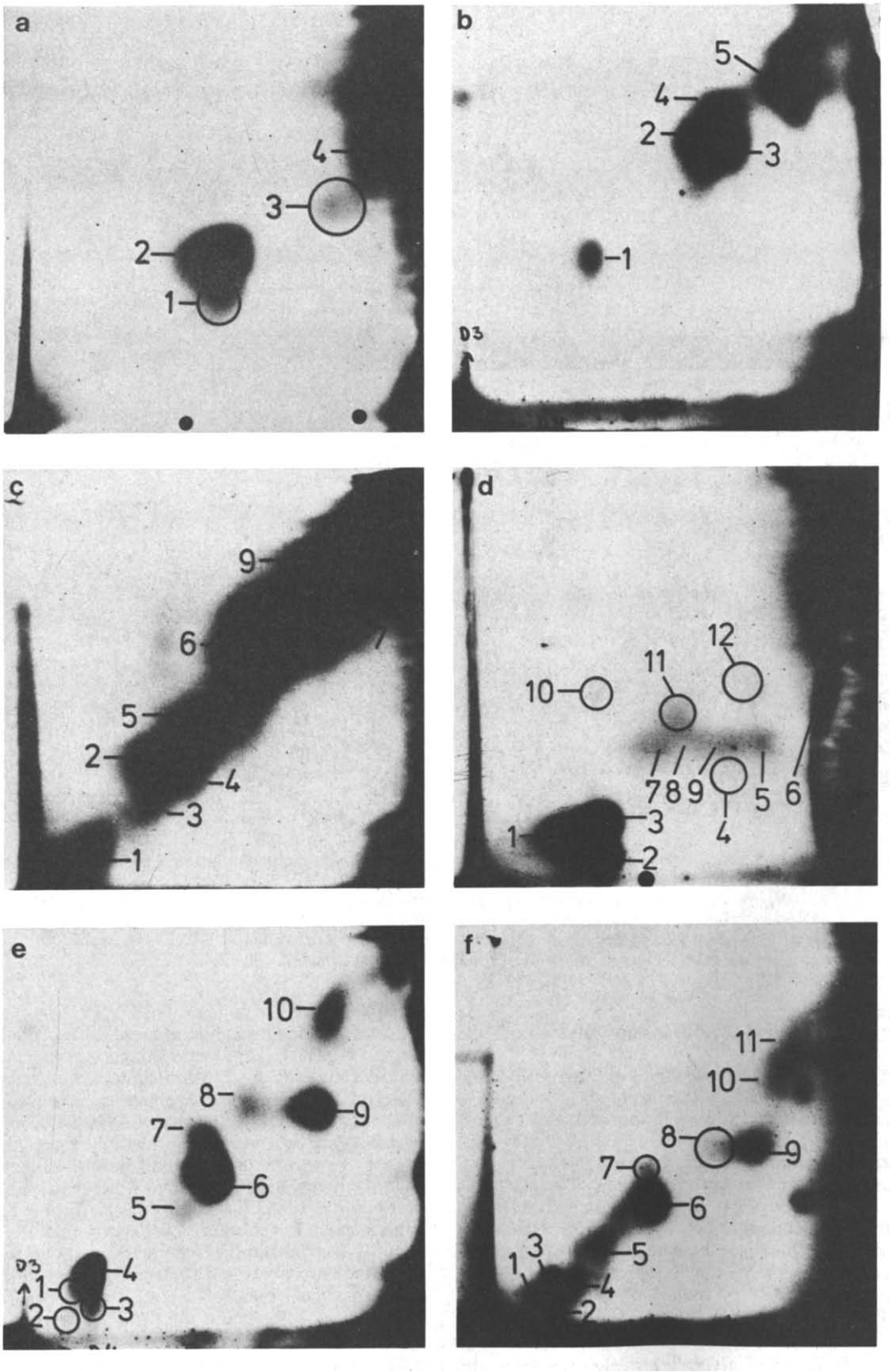



Fig. 4. Autoradiograms of DNA from HeLa $S_3$ cells treated with the derivatives of 1-nitro-9-aminoacridine, (a) control non-treated cells, (b) 2 $\mu$g/ml nitracrine, (c) 0.22 $\mu$g/ml C-857 and (d) 7.4 $\mu$g/ml C-1006. The cells were incubated with the drugs at concentrations constituting 1360 × $EC_{50}$ for 4 hr. The DNAs were isolated, digested to dNps, which were subjected to $^{32}$P-labeling and the individual adducts were resolved by TLC as described in Materials and Methods. Approximately 120 $\mu$Ci was applied to the chromatograms. Spots were visualized by autoradiography at −20° for 3 days.

applied, 1 DNA nucleotide in approximately 578 was adducted.

The map of $^{32}$P-labeled digest of DNA from HeLa $S_3$ cells treated with C-1006 displayed the most complex pattern (Fig. 4d). In addition to 9 distinct adduct spots, samples reproducibly exhibited a grey area extending from the origin region to the upper right-hand margin of the chromatograms. This grey area must have derived from diffuse not fully resolved DNA adduct spots since it was never observed in control autoradiograms. The level of DNA modification by C-1006, calculated taking into account only radioactivity of spots 1 to 9, amounted to 1 adduct per about 1180 nucleotides.

The data given above on the level of DNA modification by the compounds under study require additional explanation. The $^{32}$P-post-labeling test has been claimed by Randerath *et al.* to be a quantitative assay [e.g. 18]. During our experiments on adduct formation by 1-nitro-9-aminoacridines with DNA of HeLa $S_3$ cells, we made an observation, however, that the efficiency of $^{32}$P-labeling of DNA adducts varied considerably from preparation to preparation (data not shown). It seemed to strongly depend on the time which had elapsed between $\lambda$-[$^{32}$P]-ATP synthesis and performing the experiments. For instance, with two-week-old $\gamma$-[$^{32}$P]-ATP no labeling of DNA adducts was detected while radioactivity was still efficiently incorporated into normal nucleotides. Nevertheless, in spite of the variations in $^{32}$P-labeling of DNA adducts, their chromatographic patterns as well as their relative amounts were greatly reproducible in several independent assays. Since we had to rely on outer sup-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plies of γ-[$^{32}$P]-ATP, without always being able to complete the experiments within a week, we decided to concentrate on qualitative rather than on quantitative analysis of DNA adducts formed by nitroacridines. The estimations of the levels of DNA modification given above were calculated only from those $^{32}$P-post-labeling assays in which the level of DNA modification by nitracrine was comparable with the one obtained using this compound in isotopically labeled form. The presented results are the means of the levels of DNA modification obtained during such, considered as reliable, experiments.

In vivo *modification of DNA from Ehrlich ascites tumor cells with derivatives of 1-nitro-9-aminoacridine.* The detection of DNA adducts formed in Ehrlich ascites tumor bearing mice was carried out, similarly to HeLa $S_3$ cells, for three derivatives of 1-nitro-9-aminoacridine: nitracrine, C-857 and C-1006. Previous experiments demonstrated that the irreversible binding of [$^{14}$C]-nitracrine to DNA of Ehrlich ascites tumor cells *in vivo* reached its maximum 4 hr after single i.p. injection of 0.3 mg of the drug per mouse [8]. Being interested in high DNA adduct levels, we adopted the above conditions for our $^{32}$P-post-labeling analyses of DNA adduct formation by 1-nitroacridines. The 4 hr exposure time was thus routinely used for all the compounds studied. The doses of C-857 and C-1006 administered to mice were of equal cytotoxicity as the dose used in the case of nitracrine, and were calculated from $EC_{50}$ values (Fig. 3) under the assumption that *in vitro* and *in vivo* activities are quantitatively parallel.

Figure 1 illustrates autoradiograms obtained for DNA samples from control mouse and from animals sacrificed 4 hr after a single i.p. injection of the drugs. No deoxyribonucleotide-adducts were obtained from mice that had been exposed to saline only (Fig. 1a) while $^{32}$P-labeled digests of DNAs of Ehrlich ascites tumor cells treated *in vivo* with the derivatives of 1-nitroacridine gave rise to characteristic, highly reproducible in a number of experiments, fingerprints (Figs. 1b–d). Interestingly, the autoradiographically detected patterns of chromatographic spots closely resembled the adduct profiles obtained from experiments with HeLa $S_3$ cells. The relative amounts of adducts were also similar in the both biological systems. For example, the major nitracrine-DNA adduct in HeLa $S_3$ cells (Fig. 4b, spot 2) predominated in DNA from Ehrlich ascites tumor cells as well, constituting about 60% of the total adduct radioactivity (Fig. 1b, spot 2).

Earlier experiments, carried out with the aid of isotopically labeled nitracrine, demonstrated that the fractionation of enzymatic digest of DNA isolated from Ehrlich ascites cells exposed *in vivo* to the drug allowed for the separation of five [$^{14}$C]-nitracrine nucleoside adducts [8]. The $^{32}$P-post-labeling analysis revealed only four chromatographic spots corresponding to nitracrine-DNA adducts (Fig. 1b). This discrepancy in the number of nitracrine-DNA adducts detected by two different procedures does not seem to be due to methodological artifacts and may be explained by the intrinsic instability of DNA adducts formed by derivatives of 1-nitroacridine. This question is discussed in further sections of Results where it is shown that when DNA from HeLa $S_3$ cells treated with nitracrine was subjected to $^{32}$P-fingerprinting procedure 3 days after its isolation, five DNA adduct spots were detected (Fig. 5). It is conceivable to suspect that a similar situation may occur within DNA of Ehrlich ascites tumor cells. Thus, the previously observed elevated number of nitracrine-DNA adducts, as compared to present results, would mirror some kind of rearrangement of the adducts taking place during the time-consuming separation methodology.

*Modification of calf thymus DNA* in vitro *with derivatives of 1-nitro-9-aminoacridine.* The $^{32}$P-analysis of *in vitro* DNA adduct formation by nitracrine and C-857 was performed in two different non-cellular systems. To mimic metabolic activation, necessary for covalent binding of 1-nitroacridines to DNA, the first employed rat hepatic microsomal fraction as a source of activating enzymes. In these experiments control samples, consisting of likewise studied samples of calf thymus DNA and the drug, were incubated in the presence of either heat-inactivated microsomes or buffer alone. The second system used was even simpler, and apart from calf thymus DNA and the drug, contained DTT serving here as a reducing agent [17]. The control samples were depleted of DTT. We expected that in the latter system the formation of DNA adducts resulting from reduction of a nitro group should be favoured.

$^{32}$P-post-labeling assay demonstrated that nitracrine and C-857 were able to bind to calf thymus DNA in the both non-cellular models used. Each compound gave rise to a specific pattern of chromatographic spots representing DNA adducts (Fig. 6) that were not detected in DNA digests from control samples. Surprisingly, however, no differences in DNA adduct formation in these two systems were observed. The chromatographic profiles of nitracrine-DNA adducts (Figs. 6a and b) as well as their relative amounts were found to be identical for both the *in vitro* systems employed (Figs 6a and b). Our preliminary experiments showed that this also seems to be true for the other derivative of 1-nitroacridine, C-857.

*The analysis of DNA adduct formation by 2-nitro and 4-nitro isomers of nitracrine.* It has been reported previously that 2-nitro and 4-nitro isomers of nitra-

Fig. 5. The changes in DNA adducts chromatographic profiles during storage of isolated modified DNA: maps of $^{32}$P-labeled digests of DNA from HeLa $S_3$ cells treated with 2 μg/ml nitracrine obtained (a) immediately, (b) 3 days, or (c) 3 weeks after DNA isolation and 0.22 μg/ml C-857 obtained (d) immediately, (e) 3 days or (f) 3 weeks after DNA isolation. HeLa $S_3$ cells were treated with derivatives of 1-nitro-9-aminoacridine for 4 hr, DNAs were then isolated and frozen at −20°. At times indicated above samples of modified DNAs were assayed by the $^{32}$P-post-labeling method described in Materials and Methods. Approximately 120 μCi was applied to the chromatograms and spots were visualised by autoradiography at −20° for 3 days.

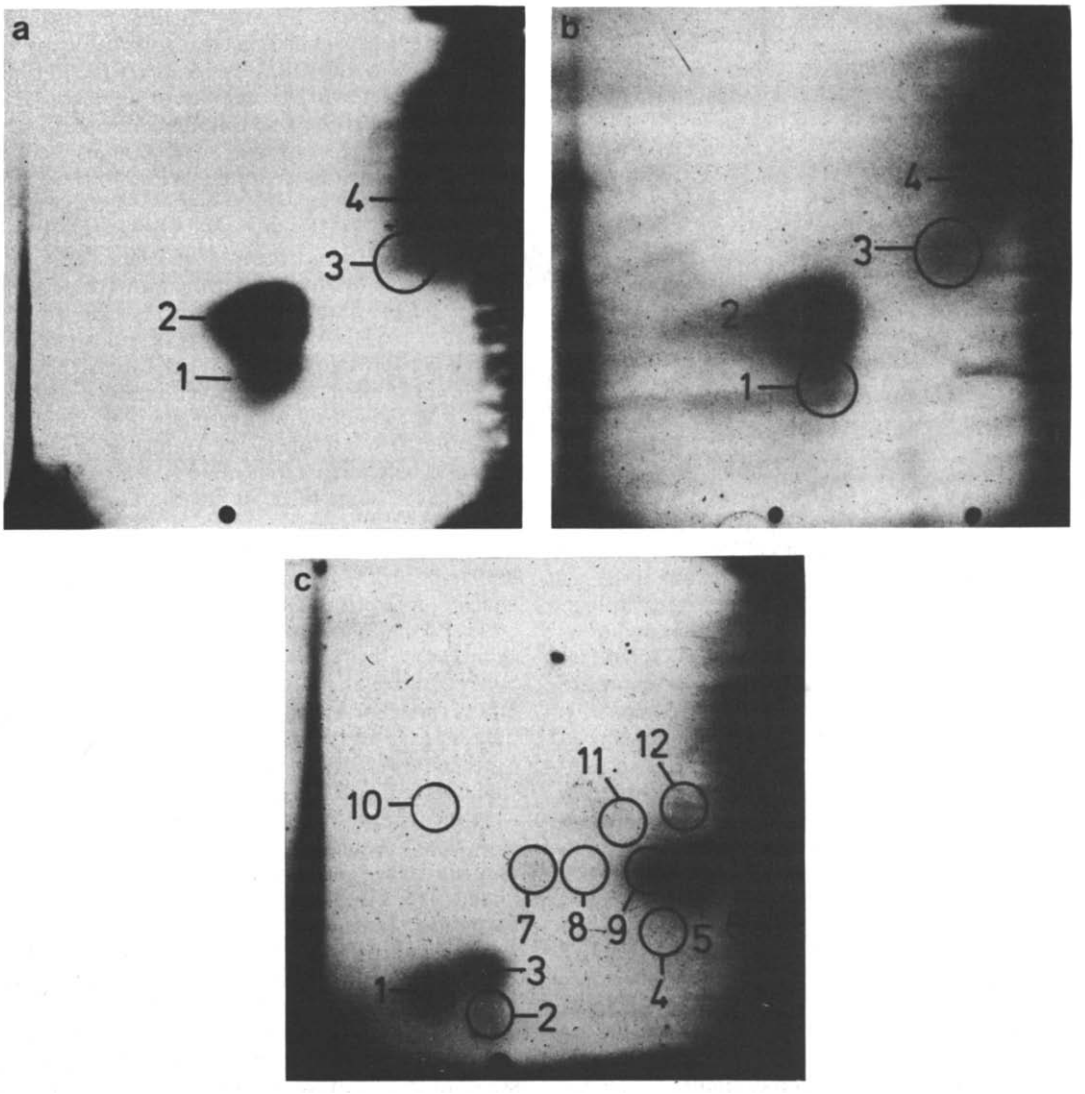


Fig. 6. Autoradiograms of calf thymus DNA modified *in vitro* with the derivatives of 1-nitro-9-aminoacridine, (a) nitracrine in the presence of microsomes or DTT (b) and (c) C-857 in the presence of microsomes. Calf thymus DNA was incubated with 50 $\mu$M of the drugs in the presence of either 1 mg/ml rat hepatic microsomal fraction for 1.5 hr or 2 mM DTT for 1 hr. The modified DNAs were isolated and submitted to standard $^{32}$P-post-labeling assay. Adducts were detected by autoradiography at $-20^{\circ}$ for 3 days.

crine are able to intercalate into DNA to a higher extent than their 1-nitro counterpart [5]. Despite their significant intercalative properties, they do not however, exhibit biological activity. Furthermore, these compounds were shown as being, contrary to 1-nitroacridines, not capable of introducing covalent interstrand crosslinks in cellular DNA [6]. All the above findings suggested a key role of the position of a nitro group in biological properties of nitroacridines. To investigate its relevance for covalent binding to DNA, we performed, in extension to the described studies on derivatives of 1-nitroacridine, $^{32}$P-post-labeling analysis of DNA adduct formation by 2-nitro and 4-nitro isomers of nitracrine.

The experiments were conducted for two biological models: HeLa $S_3$ cells in culture and Ehrlich ascites tumor bearing mice. In these assays HeLa $S_3$ cells were treated for 4 hr with 100 $\mu$g/ml of either 2- or 4-nitro isomer of nitracrine, the cellular DNAs were isolated and submitted to $^{32}$P-post-labeling procedure. It turned out that the resultant autoradiograms did not exhibit any chromatographic spots which could not be observed in control autoradiograms (data not shown). Similarly, no deoxyribonucleotide adducts were detected in $^{32}$P-labeled digests of DNAs from Ehrlich ascites cells exposed *in vivo* to 7.5 mg/mouse (the highest non-toxic to animals dose) of 2-nitro or 4-nitro isomers of nitracrine. These results showed that under conditions applied, neither 2-nitro nor 4-nitroacridine formed DNA adducts in biological models used.

*Persistence of DNA binding and stability of DNA*

*adducts formed by nitracrine and C-857.* In several papers dealing with carcinogen-DNA adduct formation it was mentioned that the modified DNAs had been isolated and stored before they were submitted to $^{32}$P-post-labeling analysis [e.g. 19, 20]. Since as far as the $^{32}$P-post-labeling assay is concerned, such an experimental schedule is very convenient, especially when larger numbers of samples are to be handled, we decided to examine whether it would give the reliable results in the case of derivatives of nitroacridine. Our experiments carried out with the aid of isotopically labeled nitracrine demonstrated that the level of covalent binding of this compound to DNA was stable for at least 9 days following DNA isolation (Fig. 2). On the other hand, however, the $^{32}$P-post-labeling analysis of DNA adducts formed by nitracrine and C-857 with DNA of HeLa $S_3$ cells revealed that although overall adduct-associated radioactivity remained unchanged up to three weeks, the adducts themselves underwent some kind of chemical rearrangement. Figure 5 presents the maps of $^{32}$P-labeled digests of DNAs from HeLa $S_3$ cells treated with either nitracrine or C-857 obtained 3 days (panel b and e) or 3 weeks (panel c and f) after DNA isolation. The $^{32}$P-fingerprints of such digests obtained using freshly isolated DNA, which were discussed also in earlier sections of Results, are given for comparison (Fig. 5, panel a and d). As it is shown, in the case of nitracrine, the number of chromatographic spots corresponding to DNA adducts depended on the time that had elapsed between DNA isolation and detection of adducts; the longer was this period the more spots were detected: four spots when $^{32}$P-fingerprinting was carried out immediately after DNA isolation, five spots and ten spots, respectively, after 3 days and 3 weeks of storing of isolated DNA at −20°.

As opposed to nitracrine-DNA adducts, the storage of isolated C-857-modified DNA from HeLa $S_3$ cells at −20° resulted in the detection of a similar number of chromatographic spots but the adduct pattern obtained 3 days after DNA isolation changed dramatically comparing to fresh DNA preparations and it was alike when C-857-DNA adducts were analysed 18 days later.

*Modification of calf thymus DNA* in vitro *with nitracrine and* C-857 *under anaerobic conditions.* In an attempt to elucidate whether the conditions of reduction of a nitro group have any influence on covalent binding of 1-nitro-9-aminoacridines to DNA, we undertook the $^{32}$P-post-labeling analysis of *in vitro* DNA adduct formation by nitracrine and C-857 in anaerobic conditions. In these only preliminary studies, calf thymus DNA was incubated with 50 μM of either nitracrine or C-857 in the presence of rat hepatic microsomal fraction under nitrogen atmosphere and subsequently subjected to $^{32}$P-post-labeling procedure. The resultant $^{32}$P-fingerprints were compared with those, hitherto shown in Fig. 6, obtained in parallel experiments in which DNA modification with respective 1-nitroacridines had been carried out under aerobic conditions. It turned out that for the both compounds, the patterns of chromatographic spots as well as the relative amounts of individual DNA adducts (data not shown) were very similar to the corresponding autoradiograms and estimations obtained for samples incubated under air. Also the extent of covalent binding of neither nitracrine nor C-857 to DNA was influenced by anaerobic, thus favouring reduction, conditions. The levels of DNA modification by the drugs, calculated from both $^{32}$P-post-labeling assays and experiments employing [$^{14}$C]-nitracrine (see Fig. 2), were comparable in samples incubated under nitrogen and under air.

## DISCUSSION

The $^{32}$P-post-labeling analysis of DNA adduct formation by three derivatives of 1-nitro-9-aminoacridine, nitracrine, C-857 and C-1006, that differ in the structure of the 9-amino side chain (for structures see Fig. 3) showed that all these compounds are indeed able to bind to DNA of Ehrlich ascites tumour cells *in vivo* as well as to DNA of HeLa $S_3$ cells in tissue culture. Each compound gave rise to a distinct pattern of chromatographic spots corresponding to DNA adducts and the respective adduct profiles were similar, if not identical, in both biological models employed. This indicates that DNA adducts formed were identical in these systems and may reflect the similarity of metabolic pathways involved in the activation of 1-nitro-9-aminoacridines in tumor cells of human and murine origin.

The selectivity of the chromatographic procedures suggests that all the detected adducts contained aromatic moieties [10]. One could suspect, however, that some of them arose with concomitant release of 9-amino side chains. Such adducts would be thus common for all 1-nitro-9-amino derivatives studied. The autoradiograms presented in this paper did not allow us to ascertain whether it is the case. It could not also be decided whether any of the adducts resulted directly from DNA crosslinking.

While examining autoradiograms obtained it was interesting to note that the number of DNA adducts formed by a given 1-nitro derivative could be correlated with neither its cytotoxic activity against HeLa $S_3$ cells nor complexity of its chemical structure. The most active compound, C-857, gave rise to at least 12 DNA addition products, less cytotoxic nitracrine to 4, while C-1006 exhibiting the lowest activity displayed the most complex adduct pattern. This last finding could be explained by the fact that C-1006 possesses the most complicated 9-amino side chain with an additional amino group in position 3′ (see Fig. 3). Such a 3′-amino group can be metabolized, bind to DNA and thereby give rise to still new adducts. However, nitracrine also contains this 3′-residue, albeit chemically simpler, yet it forms less DNA adducts than C-857 which is devoid of it entirely. Nevertheless, since all the 1-nitro derivatives studied have the same nitro-substituted acridine core, it must be the structure of the side chain that is responsible for the diversity of DNA adducts formed by individual 1-nitro-9-aminoacridines. All the same, the above example illustrating the differences in DNA adduct formation by a group of closely related compounds demonstrated that it might be difficult to predict the number of DNA modifications introduced by any chemical mutagen solely on the basis of its chemical structure.

The most striking dependence of activity on the chemical structure of the parent compound within the group of nitroacridines studied here is seen by comparing the isomers of nitracrine that differ in the position of a nitro group. No DNA adduct formation was detected in the two cellular systems used with 2-nitro and 4-nitro substituted 9-aminoacridine and the compounds are biologically inactive, while 1-nitro substituted nitracrine exhibited high DNA binding and shows significant cytotoxic activity. This finding points out the relevance of the position 1 of a nitro group for both covalent binding to DNA and cytotoxic activity. Such a substitution of an acridine core was also demonstrated to be necessary for other activities of 1-nitro-9-aminoacridines. These included: DNA crosslinking [6], inhibition of precursors' incorporation [5, 21, 22], induction of DNA repair synthesis [23]. All these processes were not affected by derivatives containing a nitro group in other positions of the 9-aminoacridine ring. Furthermore, the above listed activities exerted by 1-nitro-9-aminoacridines were found to be linearly correlated with the growth inhibitory activity of these agents [9, 21, 22, 23]. Similar correlation between cytotoxic activity and the level of DNA modification by 1-nitro derivatives studied was observed in the present work. When the HeLa $S_3$ cells were treated with the three drugs at the concentrations constituting the same multiplicity of $EC_{50}$ ($1360 \times EC_{50}$ in our experiments), the degree of covalent binding to DNA remained comparable for all the compounds and amounted to 1 molecule of a drug per 485, 578 and 1180 nucleotides for nitracrine, C-857 and C-1006, respectively. Thus, at equitoxic concentrations of 1-nitro derivatives the frequency of total DNA adducts was approximately equal. 2-Nitro and 4-nitro isomers of nitracrine did not form DNA adducts and are biologically inactive in spite of the fact that they intercalate into DNA even better than their 1-nitro counterpart [5]. Taken together, all these data strongly suggest that it is covalent binding and DNA crosslinking which are prerequisite for biological activity of 1-nitro-9-aminoacridines while intercalation into DNA seems to be of negligible, if any, biological significance.

$^{32}$P-post-labeling assay demonstrated that nitracrine and C-857 were also able to bind covalently to calf thymus DNA in a non-cellular system in which the metabolic activation of the drugs was accomplished by the addition of a rat hepatic microsomal fraction. In an attempt to elucidate the influence of the conditions of a nitro group reduction on subsequent DNA adduct formation, we additionally carried out experiments in this system under anaerobic conditions or used DTT as a reducing agent in place of microsomes. Surprisingly, no differences in DNA adduct formation by these two 1-nitro derivatives were detected in all the non-cellular systems applied. The chromatographic patterns of adducts as well as their relative amounts were identical, only in the case of the system employing DTT the overall binding of nitracrine (for C-857 not determined) was about 4 times higher; 1 molecule of [$^{14}$C]-nitracrine per 97 nucleotides as compared to 385. The notably lower level of DNA modification by nitracrine in the systems utilizing microsomes may be due to competitive binding of the drug to microsomal proteins that apparently cannot occur in samples incubated with DTT.

Wilson *et al.* demonstrated that nitracrine is selectively toxic to Chinese hamster ovary cell line AA8 under hypoxic conditions in culture [24], so the unchanged level of DNA modification by 1-nitroacridines in a non-cellular system under anaerobic conditions has come as a surprise. This result accords, however, with previous reports where the extent of irreversible binding of [$^{14}$C]-nitracrine to microsomal proteins *in vitro* was shown to be even smaller under nitrogen atmosphere than under air [7]. The metabolic activation of 1-nitro-9-aminoacridines involves both oxidative and reductive pathways and it seems that reduction of nitro group might not be the only determinant of *in vitro* binding of these compounds to DNA as well as to microsomal proteins. One possible explanation of this event would be that hydroxylamine group arising from the reduction of 1-nitro group requires additional activation that cannot be accomplished in non-cellular circumstances. The other would rely on similarities of the metabolic activation of 1-nitroacridines and that of some nitro-substituted polycyclic aromatic hydrocarbons. For instance, it has been established that oxidized metabolites of 1-nitropyrene are reduced to DNA binding derivatives more easily than 1-nitropyrene itself [25, 26]. If this also held for 1-nitroacridines, they could be expected to undergo nitroreduction more readily after former oxidation which in turn is more effective under aerobic conditions. So the conducive to reduction circumstances would not necessarily lead to increased formation of DNA reactive species and, as it was observed in our experiments, no enhancement of DNA modification would take place. The clarification of this phenomenon will require further investigation.

Even more unexpected was the finding that samples in which modification of DNA by 1-nitroacridines had been conducted using DTT gave rise to the same profiles of DNA adducts as those obtained in other systems. These reactions were carried out without any agent(s) that could account for the oxidation of the drugs, so oxidized metabolites as well as respective drug-modified nucleotides were unlikely to occur. However, it has been reported recently that 1-nitro-9-aminoacridines are able to bind to nucleosides *in vitro* by nucleophilic substitution reaction that involves the 9-amino side chain [27]. This also could explain *in vitro* DNA crosslink formation by nitracrine in the presence of sulfhydryl compounds [17].

Our studies on DNA adduct formation by nitroacridines performed by means of $^{32}$P-post-labeling assay allowed some observations concerning the method itself to be made. First, quantitative measurements of DNA adduct levels were poorly reproducible with concomitant very good replicability of patterns of chromatographic spots (in four to eight independent assays). Hence, it was assumed that the discrepancies in quantitative data might be attributed to greater variations in the $^{32}$P-labeling of adducts as compared to normal nucleotides. We noticed that indeed, when applying the

same amount of radioactivity, the "older" γ-[$^{32}$P]-ATP was to the lesser extent [$^{32}$P]-phosphate was incorporated into DNA adducts while labeling of normal nucleotides was only slightly affected (data not shown). So it can be speculated that during radiolysis of γ-[$^{32}$P]-ATP some unknown substances arose that preferentially inhibited labeling of the adducts. The accuracy of quantitation of DNA adducts by $^{32}$P-post-labeling assay has been also questioned by other authors who demonstrated that the level of DNA adducts formed by some aromatic carcinogens determined by post-labeling with $^{32}$P was usually 50 to 80% of the value obtained for tritiated adducts resolved by HPLC [e.g. 28]. The influence of the quality of γ-[$^{32}$P]-ATP on efficiency of polynucleotide kinase catalysed $^{32}$P-labeling of DNA adducts has not been reported to date.

The other observation refers to intrinsic instability of DNA adducts formed by 1-nitroacridines under study during storage of isolated DNA samples. In some papers dealing with $^{32}$P-postlabeling analysis of DNA adduct formation it has been mentioned that after isolation modified DNAs were stored frozen until submitted to further experimental procedure [e.g. 19, 20]. It has not been commented, though, whether any changes in DNA modifications occurred, if at all, after the storage period as compared to fresh DNA preparations. In the case of 1-nitro-9-aminoacridines the overall level of binding in isolated DNA was found out to be stable within three weeks as revealed by both $^{32}$P-post-labeling assay and measurements obtained with the aid of [$^{14}$C]-nitracrine. The former technique showed however, that the adducts formed by nitracrine as well as C-857 with DNA of HeLa $S_3$ cells were undergoing some kind of chemical rearrangement when isolated modified DNAs were kept at −20°. The maps of $^{32}$P-labeled digests of nitracrine- and C-857-modified DNAs exhibited different patterns of chromatographic spots for assays carried out immediately, three days and three weeks after DNA isolation. This altered behaviour of DNA adducts in chromatographic solvents used suggests that their chemical structures must have been somehow altered as well. What kind of chemical reactions are involved in this process presents an as yet unresolved question but the unchanged levels of overall DNA binding of 1-nitroacridines imply that the presumptive rearrangement(s) did not lead to release of the compounds from DNA. In summary, the above methodological observations indicate that: first, the level of modification by a given drug determined by $^{32}$P-post-labeling assay may be underestimated due to several factors [see above and 28] and generally should be considered as being rather a good estimate than an exact value. Second, the $^{32}$P-post-labeling analysis of DNA adducts formed by a given chemical should be preferably conducted using freshly isolated DNA samples in order to avoid any possible decomposition of adducts. Being unaware of such a possibility one could easily misinterpret the obtained results. For instance, the autoradiograms presented here from experiments with nitracrine- and C-857-modified DNAs carried out three weeks after their isolation, displayed quite similar adduct patterns. This could be misinterpreted as indicative for the general similarity of structures of DNA adducts formed by these two compounds, while the assays performed using fresh DNA preparations proved it not to be the case.

Covalent modification of DNA has drawn much attention recently since such reactions are believed to be related to the cytotoxic action of several antineoplastic agents [29] as well as to be crucial to the initiation of the carcinogenic process [30 and references therein]. The requirement of radioactive test chemicals had long hampered DNA binding studies by restricting them to those DNA-reactive agents that were readily available in isotopically labeled form. The $^{32}$P-post-labeling assay developed by Randerath *et al.* [10], that does not require radioactive test chemicals, has become a valuable tool in the detection of aromatic carcinogen-DNA adducts. Surprisingly, apart from applying this method to the detection of DNA adducts formed by mitomycin C [31], whose covalent binding to DNA was demonstrated long ago [32] as well as DNA adduct structures determined [e.g. 33], to date this method has not been employed for analysis of DNA adduct formation by antineoplastic agents. The study presented here exploited $^{32}$P-post-labeling test to investigate the mechanism of action of derivatives of 1-nitro-9-aminoacridine, a new class of antitumor agents. The analysis of DNA adduct formation by some closely related nitroacridines whose chemical structures differed in either the structure of the 9-amino side chain or in the position of a nitro group support further the hypothesis that the ability to bind covalently to DNA and to induce DNA covalent crosslinks, as opposed to intercalation, underlies the cytotoxic and antitumor activity of 1-nitro-9-aminoacridines.

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