Experimental Cell Biology
Editors: J.R. Rüttner, Zürich; F. Deinhardt, München; A. Wolsky, New York, N.Y.; H. Ramseier, Zürich; L. Helson, New York, N.Y.
Publishers: S. Karger, Basel
Reprint (Printed in Switzerland)

Expl Cell Biol. *49:* 220–231 (1981)

Correlation between in vitro DNA Synthesis, DNA Strand Separation and in vivo Multiplication of Cancer Cells

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Key Words. 9,10-Dimethylbenzanthracene · 1-(2-Chloroethyl-1)-nitrosourea · Dimethylsulfoxide · Actinomycin D · Daunorubicin · Croton oil · DNA synthesis · DNA strand separation · Cancer cells · Steroid

Abstract. The chemicals 9,10-dimethylbenzanthracene (DMBA), ethionine, daunorubicin, actinomycin D, 1-(2-chloroethyl-1)-nitrosourea (CCNU), steroids, croton oil and dimethylsulfoxide (DMSO) were used in order to correlate their effect on the in vitro synthesis of normal and cancer DNA, on DNA strand separation and on accelerated in vivo multiplication of cancer cells. All of the compounds tested strongly stimulate the synthesis of cancer DNA in vitro catalyzed by DNA-dependent DNA polymerase I and measured as an acid-precipitable labeled product. Under the same conditions, the synthesis of DNA originating from healthy tissues is only slightly enhanced, except in the case of croton oil and DMSO. These substances are almost equally active on cancer and normal DNA. Although both cancer and normal DNA contain a large amount of double-stranded regions, the extent of DNA strand separation measured by the increase in UV absorbance (hyperchromicity) in the presence of each compound tested is much higher for all cancer DNA than for corresponding normal DNA. In contrast, DMSO and croton oil do not appear to distinguish cancer DNA from normal DNA. Additive and differential effects of various compounds on cancer DNA strand separation can be observed. Small doses of DMBA and CCNU stimulate the multiplication of Ehrlich ascites tumor cells in vivo in mice. There is thus a possible correlation between DNA strand separation, DNA synthesis, multiplication and differentiation of cancer cells in the presence of the above compunds, which is different from the response of normal cells to these compounds.

Introduction

Normal cell growth and division process (at given intervals) involves physiologically regulated unwinding and separation of DNA double strands in order to allow access to DNA and RNA polymerases. Accelerated multiplication as well as differentiation of normal and cancer cells require highly increased DNA, RNA and protein synthesis whose initial step is DNA strand separation. Using the Oncotest [2] for the screening of

carcinogenic compounds, we have shown that carcinogens strongly stimulate in vitro cancer DNA synthesis, but only slightly stimulate normal DNA synthesis. Low concentrations of carcinogens can also stimulate the multiplication of cancer cells in animals [1, 3] and plants [5, 20]. On the other hand, it has been reported that actinomycin D [21] and tumor-promoting agents such as phorbol derivatives induce terminal differentiation in promyelocytic leukemia cells [18, 21] when used at very low concentrations. Dimethylsulfoxide (DMSO) which is teratogenic in animals [8, 12], also induces erythroid cell differentiation in murine-virus-infected erythroid leukemia cells [14, 28] as well as terminal differentiation of human promyelocytic leukemia cells [10]. Similar results were obtained with steroids [28]. Although morphological, biological and immunological changes induced by phorbol derivatives or DMSO have been characterized, no precise mechanism of action has been proposed.

In order to account for enhanced in vitro cancer DNA synthesis [2] and accelerated multiplication of vegetable [5, 20] and mammalian [1, 3, 21] cancer cells in the presence of carcinogens, anticancer drugs or various other compounds such as DMSO, croton oil and steroids, we attempted to establish possible correlations between in vitro cancer DNA synthesis, in vivo cancer cell multiplication and in vitro DNA strand separation catalyzed by the above compounds.

Materials and Methods

The following reagents were used: pancreatic RNase A and RNase T_1 (Worthington Inc., N.J., USA): 9,10-dimethyl-1,2-benzanthracene (DMBA; Nutritional Biochemical Co., Cleveland, Ohio, USA); dl-ethionine (Hoechst, Paris, France): Lomustine (1-[2-chloroethyl]-3-cyclohexyl-1)nitrosourea; (CNU; Bellon, Paris, France); 5-FU (Hoffmann-La Roche, Basel, Switzerland); daunorubicin (gift from Dr. *R. Maral*, Rhône-Poulenc, Ivry/S, France); estradiol and testosterone (gifts from Dr. *Ray*, Institut Pasteur, Paris, France); lauryl sulfate (Serlabo, Paris, France); DMSO (Merck, FRG); croton oil (Corporation pharmaceutique française, Melun, France); actinomycin D (Merck, Sharp & Dohme, Rahway, N.J., USA).

Animals: Swiss mice (Ifa Credo, Orleans, France).

Origin of DNA: after excision, healthy and cancerous human tissues (breast, lung, neurocarcinoma), cancer cells (Ehrlich ascites tumor cells) or normal tissue from monkey brain, mouse spleen or duck spleen were frozen at −20 °C and then gently broken down in 2 SSC solution. DNA was extracted first using phenol and then chloroform in the presence of lauryl sulfate, as previously described [2, 26]. RNA, which contaminates DNA preparations, was practically eliminated by incubation with RNase A and T_1 RNase (20 μg and 10 units/ml, respectively) for 30 min at 36 °C in 1 SSC solution. RNase was then removed by several chloroform treatments, each of which was followed by centrifugation in an SSI Sorval centrifuge (5,000 *g* for 10 min). DNA was precipitated with 2 vol of 96% alcohol, dissolved in 2 SSC solution, and dialyzed against this same solution for 24 h at 4 °C. Purified DNA (absorbance at 260/280 = 2.1) was stored at −20 °C for several months without losing its polymerized form. Before use, DNA was dialysed against distilled water for 2 h at 4 °C in order to eliminate salts which could interfere in the experiments to be carried out.

Characterization of DNA

The RNA content, determined by the orcinol reaction [4], is lower than 10%. Protein content makes up less than 1.0% [22]. The hyperchromic effect on incubation with NaOH is 30–42% for the DNA used. In the ultracentrifuge or in an alkaline sucrose gradient the material forms an essentially homogeneous peak ranging between 26 and 36 S and a small one of 2.5–4.2 S.

Isolation of DNA-Dependent DNA Polymerase I (EC 2.7.7.7)

Since DNA-dependent DNA polymerase I from *Escherichia coli* synthesizes DNA by the same mechanism as the corresponding enzyme from mammalian

tissues, we used this enzyme for the in vitro DNA assay system [2]. The enzyme was partly purified by ammonium sulfate precipitation and by two passages in a DEAE-cellulose column as previously described [4]. The enzyme preparation 280/260 ratio should range between 1.5 and 1.7.

In vitro DNA Synthesis

The incubation conditions for in vitro DNA synthesis have been described elsewhere [2]. The amount of acid-precipitable ^{3}H-labeled DNA (TCA 5% solution) was determined in the absence or presence of the compound to be tested. The acid-precipitable product was filtered on a Millipore GF/C glass filter, washed and dried. Its radioactivity was then measured with a Packard liquid spectrometer.

UV Absorbance of DNA

DNA was dissolved in a 0.01 *M* Tris-HCl buffer solution (pH 7.65). The substances to be tested were dissolved in 10 µl of the same buffer and were then added to the blank and the DNA solution. The mixture was gently agitated at room temperature and its UV spectrum determined. The concentrations inducing the maximal increase in UV absorbance at 260 nm were determined. UV absorbance was also measured at pH 7.60, 7.70. 7.75, 7.80 and 7.95.

Stimulation of Ehrlich Ascites Tumor Cells in Mice by DMBA or CCNU

Mice were infected with 10^5 Ehrlich ascites tumor cells by intramuscular injection (i.m.). One group served as controls and the other group was treated with low doses of DMBA, administered daily to the same inoculation site for 5 consecutive days. The mice were weighed at various intervals (indicated on fig. 10) and on the 20th day they were sacrificed. After excision, the tumors were weighed. For treatment with CCNU, Ehrlich ascites tumor cells (10^4/mouse) were inoculated intraperitoneally (i.p.). One group served as controls and the other group was treated i.p. with 200 µg every other day, over 15 days.

Results

In the present study, we have investigated three effects of the substances tested: (1) in vitro synthesis of normal and cancer DNA; (2) in vitro strand separation of both types of DNA, and (3) stimulation in vivo of tumor cell multiplication.

In vitro Synthesis of Cancer and Normal DNA

Dose-response curves are illustrated in figure 1. DMBA, ethionine and CCNU strongly stimulate the in vitro synthesis of Ehrlich ascites tumor DNA, while these substances exhibit only a slight enhancing effect on normal DNA synthesis. For actinomycin D and daunorubicin, there is a strong stimulation of breast cancer DNA synthesis with a linear increase in the first portion of the curve followed by a decrease in DNA synthesis, which indicates that at high concentrations these compounds prevent DNA-dependent DNA polymerase from exerting its polymerizing activity. Both actinomycin D and daunorubicin only slightly enhance the synthesis of normal breast DNA. Ethionine also enhances cancer DNA synthesis and poorly that of normal DNA, while l-methionine is completely inert. When cancer DNA from breast tissues (a steroid hormone target tissue) was used as template, testosterone and estradiol, at doses higher than physiological concentrations, stimulated DNA synthesis, which is more important in the presence of testosterone (fig. 1). In this respect, normal breast DNA responds weakly to both steroids. When we used cancer and normal DNA from lung tissues or other tissues (not steroid hormone target tissues), the stimulating action of steroid hormones, although detectable, was far less effective than with breast cancer DNA except for neurocarcinoma DNA whose in vitro synthesis is strongly stimulated by testosterone. Although different in nature, all steroids and carcinogens exhibit a common effect. They strongly stimulate cancer DNA

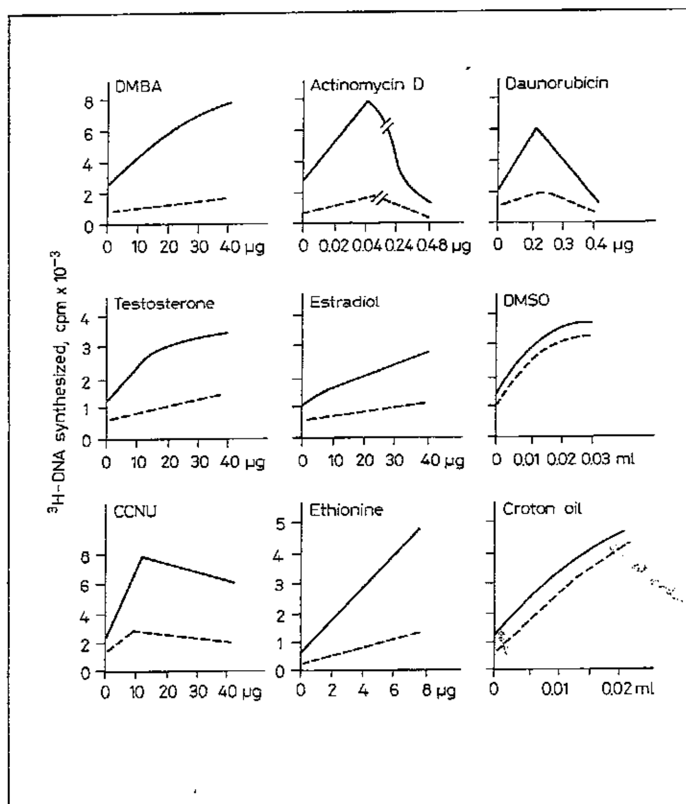


Fig. 1. Cancer and normal in vitro DNA synthesis. Effects of various compounds. Dose-response curves represent cancer DNA synthesis (——) and normal DNA synthesis (– – –). Further explanation in text.

in vitro synthesis. In contrast to carcinogens, drugs and steroids, croton oil and DMSO are almost equally effective on both cancer and normal DNA, as shown in figure 1.

DNA Strand Separation in the Presence of Carcinogens or Other Compounds

In the double DNA helical structure, DNA strands are maintained by hydrogen bonds which can be broken at a relatively high alkaline pH, resulting in an increase in UV absorbance. As shown in figure 2, there is no increase in UV absorbance when cancer DNA or normal DNA is incubated alone at pH 7.60, 7.70 or 7.80. However, beyond pH

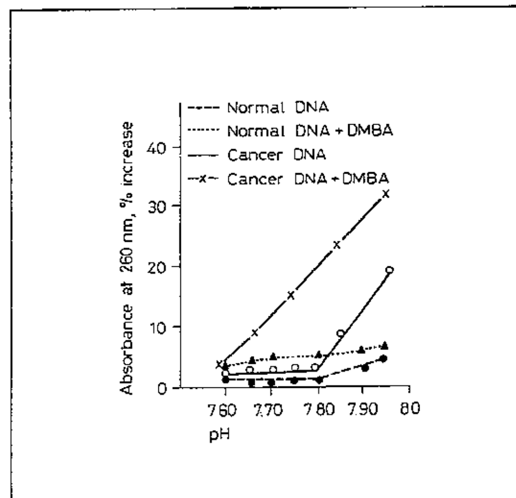


Fig. 2. Cancer and normal DNA strand separation at different pH values. Further explanations in text.

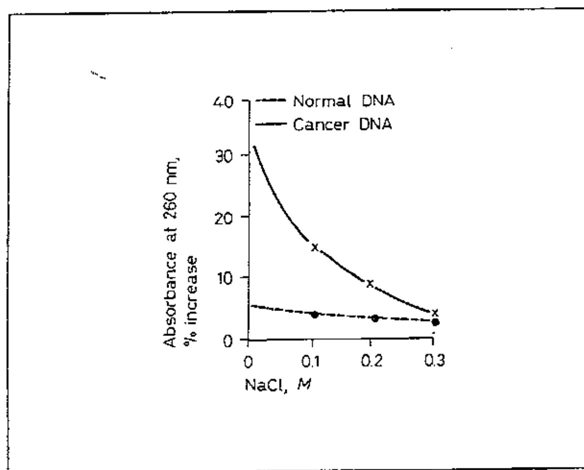


Fig. 3. Cancer (——) and normal (– – –) DNA strand separation in the presence of DMBA. Effect of ionic strength. Ehrlich ascites-tumor DNA (20 μg) and normal mouse or monkey spleen DNA (20 μg/ml) were each dissolved in 1 ml of 10^{-2} *M* HCl-Tris buffer (pH 7.65) containing various concentrations of NaCl as indicated. For each NaCl concentration, 10 μg of DMBA were used. The absorbance was measured at 260 nm. The DNA samples were read against a blank cuvette containing NaCl at given concentrations and 10 μg of DMBA/ml.

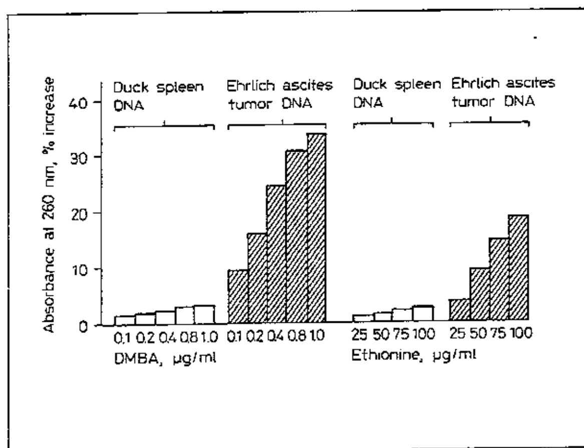


Fig. 4. Ehrlich ascites/tumor DNA and spleen DNA strand separation. Effect of DMBA or ethionine. UV absorbance of DNA was measured in the absence or presence of each compound.

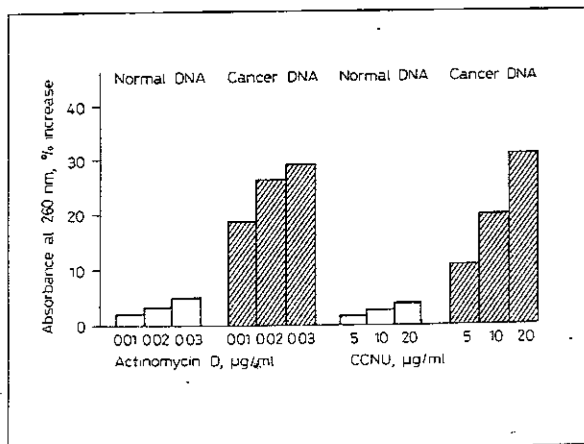


Fig. 5. Cancer and normal DNA strand separation. Effects of actinomycin D and CCNU. The effect of actinomycin was determined on human neurocarcinoma DNA and normal monkey brain DNA. The effect of CCNU was determined on Ehrlich ascites tumor DNA and normal mouse spleen DNA.

Table I. Comparison of the effects of daunorubicin, dl-ethionine and l-methionine on cancer DNA strand separation

DNA	Absorbance at 260 nm, % increase		
	daunorubucin	l-methionine, 100 µg	dl-ethionine, 100 µg
Breast cancer DNA, 20 µg[1]	21	0	13
Breast cancer DNA, 20 µg[2]	20	0	–
Ehrlich ascites tumor DNA, 20 µg	19	0	17

[1] 4 µg daunorubicin added.
[2] The daunorubicin-treated DNA (footnote 1) was dialyzed (see text) and then reincubated with 4 µg daunorubicin and l-methionine, respectively.

7.85, there is a strong increase in UV absorbance for cancer DNA but not for normal DNA. In the presence of DMBA (10 µg/ml), cancer DNA undergoes complete DNA strand separation while strand separation of normal DNA is weakly increased (fig. 2). Ionic strength plays an important role in this phenomenon (fig. 3). NaCl prevents hydrogen bond breakage by carcinogens.

As shown in figure 4, a progressive increase in UV absorbance of human breast cancer DNA dissolved in 0.01 *M* Tris buffer (pH 7.65) is induced by increasing concentrations of DMBA which, also in the case of Ehrlich ascites tumor DNA, can lead to a 35% hyperchromic effect while, in the case of duck or monkey spleen DNA, i.e. normal DNA, this effect is about 3%.

Ethionine, a potent carcinogen used at rather high concentrations, also induces separation of DNA strands isolated from Ehrlich ascites cells with a maximal increase in hyperchromicity which is of the order of 17% while this increase is about 3% in the cae of duck or monkey spleen DNA; l-methionine has no effect at all in this respect (table I).

Known as anticancer drugs and also known as carcinogens [11, 27, 29], CCNU or actinomycin D induce a strong separation of the strands of cancer DNA and act weakly on normal DNA (fig. 5). DMBA, ethionine, CCNU and actinomycin D separate to a different degree the strands of a given cancer DNA (fig. 4, 5), thus producing results which sugest the existence of different reactive sites on a given cancer DNA. These differences might be responsible for the difference in kinetics and extent of cancer DNA strand separation induced by different substances. When human breast cancer DNA was incubated in the presence of increasing concentrations of either estradiol, DMSO, testosterone or daunorubicin (fig. 6) we observed that estradiol produces a weak increase in UV absorbance while testosterone or daunorubicin is more efficient (with different kinetics) and DMSO is in between (fig. 6). These results suggest that breast cancer DNA possesses different reactive sites whose nature is not yet known.

To verify that a compound such as daunorubicin, which, at a given concentration, in-

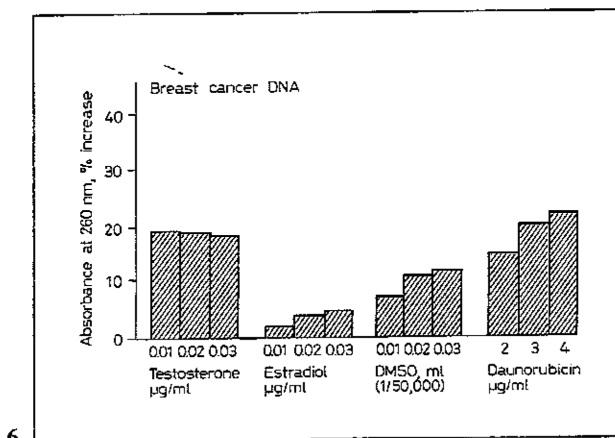
duces breast cancer DNA strand separation with a hyperchromic effect of 20% does not provoke DNA chain breaks, daunorubicin-treated DNA was dialyzed against 2 SSC solution for 24 h (with two changes) at 4 °C and finally for 2 h against distilled water. Reincubation of such DNA with daunorubicin resulted in DNA strand separation corresponding to the same hyperchromicity (20%) as that obtained with freshly prepared breast cancer DNA (table I). In contrast, normal breast DNA reacts poorly with all of these compounds, as judged by a slight increase (3%) in hyperchromicity. These results suggest that some compounds may induce the occurrence of destabilized regions in the double helical structure of DNA and consequently modify either the replication or transcription of DNA.

The effects of testosterone or estradiol on DNA strand separation are of particular interest. In fact, testosterone strongly increases UV absorbance in breast cancer DNA but not in other types of DNA, normal or cancer-related. However, human neurocarcinoma DNA appears to be an exception. As shown in figure 7, estradiol-induced slight neurocarcinoma DNA strand separation can be further increased by successive additions of testosterone, 5-FU and daunorubicin. This, finally, leads to a 30% increase in UV absorbance, which represents a very high degree of DNA strand separation. These results indicate that the compounds tested here do not interact with the same binding sites on DNA and that each of the various compounds can contribute differently to DNA strand separation. The physiological state of a cell, depending on its environment, may lead to the binding of different molecules to DNA and thus sensitize the DNA to different exogenous compounds.

The effects of croton oil and DMSO on DNA strand separation showed the same characteristics as their effect on in vitro synthesis of DNA, i.e. they did not distinguish between normal and cancer DNA. Figure 8 shows that increased concentrations of freshly prepared croton oil solution induce a progressive increase in the hyperchromicity of both human neurocarcinoma DNA and healthy brain DNA. In both cases, there is a 40% increase in UV absorbance, while the increase observed in the presence of alkali is of 42%. In addition, strand separation in healthy monkey spleen DNA, partially induced by croton oil, can be further increased by the addition of DMBA. It should be noted that DNA strand separation induced by croton oil is quite low at pH 7.60 but is highly visible at pH 7.65 or 7.70. Increased concentrations of freshly prepared DMSO also increase the UV absorbance of both cancer and normal DNA (fig. 9).

Carcinogenic Substances Stimulate in vivo the Multiplication of Ehrlich Ascites Tumor Cells

The third aim of our studies was to correlate the in vitro observations on those carcinogens that are able to stimulate cancer DNA synthesis in mice and thus to accelerate cancer cell multiplication. As a matter of fact, increased in vitro DNA synthesis should lead to stimulation of in vivo cancer cell multiplication in the presence of these carcinogens. We have tested the effect of DMBA and CCNU on tumor development in mice inoculated with Ehrlich ascites tumor cells. One group of mice received low doses of DMBA in the intramuscular inoculation site for 5 consecutive days. As shown in figure 10, the average weight of DMBA-treated mice rapidly increased compared to controls. On the



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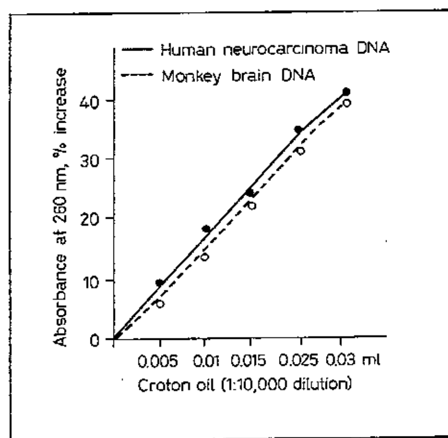

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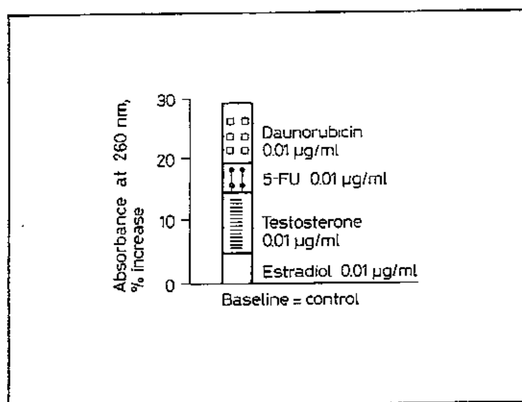

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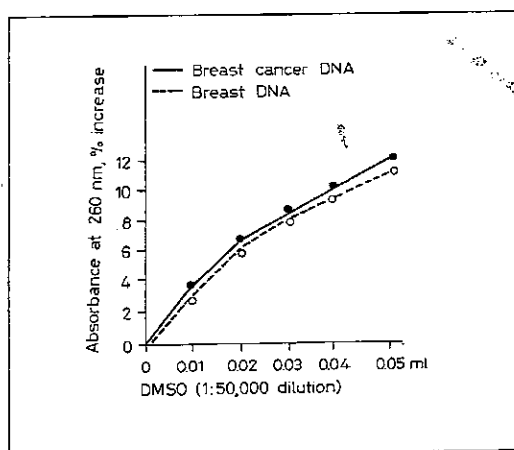

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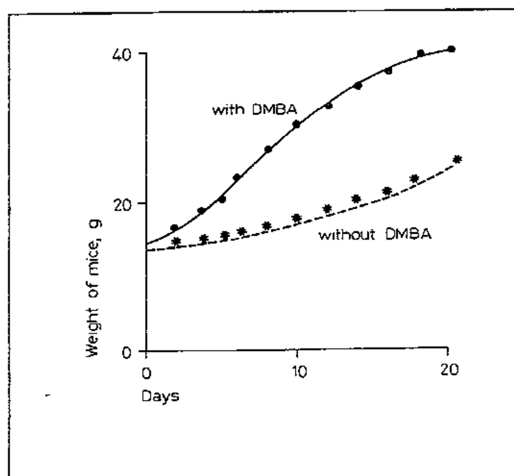
Fig. 6. Human breast cancer DNA strand separation. Effects of estradiol, testosterone, DMSO and daunorubicine. A freshly prepared solution of each compound was used. The DNA samples in the presence of each compound were read against a blank cuvette filled with 10^{-2} *M* Tris-HCl buffer (pH 7.65) containing the compound tested at the indicated concentrations.

Fig. 7. Human neurocarcinoma DNA strand separation. Effects of estradiol, testosterone, 5-FU and daunorubicin. Absorbance at 260 nm of human neurocarcinoma DNA (20 µg), dissolved in 1 ml of 10^{-2} *M* Tris-HCl buffer (pH 7.65).

Fig. 8. Human neurocarcinoma and monkey brain DNA strand separation: effect of croton oil.

Fig. 9. Human breast cancer and normal DNA strand separation: effect of DMSO.

Fig. 10. Stimulation of Ehrlich ascites cells in mice by DMBA. Further explanation in text.



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20th day, the mean tumor weight of control mice was 4.5 ± 0.03 g and that of DMBA-treated mice was 10.8 ± 0.5 g. This experiment was duplicated with CCNU, but in this case mice were inoculated i.p. The mean weight increase of CCNU-treated mice (100 μg CCNU/mouse administered i.p. every other day over a period of 15 days) was 10.5 g and that of control mice was 6 g.

These experiments demonstrate a good correlation between the stimulating effect of DMBA or CCNU on in vitro cancer DNA synthesis, DNA strand separation and the in vivo multiplication of cancer cells.

Conclusion and Discussion

From the results obtained, it is obvious that, under the same experimental conditions, most of the compounds used intervene much more efficiently in cancer DNA synthesis than in the synthesis of normal DNA. This shows that these compounds, at the concentrations used, do not modulate the activity of DNA-dependent DNA polymerase but that some molecular mechanisms must exist by which these compounds interact particularly with cancer DNA and thus lead to the increase or decrease of DNA synthesis. This raises the question: why do the compounds tested so greatly increase the in vitro synthesis of cancer DNA but only slightly that of normal DNA? DNA synthesis in vitro appears to be correlated with in vitro DNA strand separation. Both processes are dose-dependent and, for some compounds, there are optimal concentrations above which there is no further increase in in vitro DNA synthesis or in vitro DNA strand separation. The extent of DNA strand separation depends not only on the ionic strength and pH but also on the nature and concentrations of the compound used. For instance, in the presence of DMBA or CCNU, strand separation of Ehrlich ascites tumor DNA can be practically complete while, under the same experimental conditions, it is much less pronounced in the presence of ethionine or daunorubicin. These results show that an interaction between the above compounds and cancer DNA takes place that results in the break of hydrogen bonds which maintain the double strands of DNA. This observation and the fact that the strands of cancer DNA once separated by daunorubicin (20% increase in hyperchromicity) can reassociate and then separate again to the same extent show that no DNA chain breaks (covalent linkage) occurred under our experimental conditions. This conclusion is strengthened by the observation that l-methionine, which is not carcinogenic, has no hyperchromic effect either on cancer DNA or on normal DNA. From these data we concluded that carcinogenic compounds, which exhibit only a slight effect on the DNA originating from various healthy tissues, recognize cancer DNA much better than normal DNA. In this respect, the effect of steroids apears to be more restricted than that of known carcinogens. Here we showed that testosterone, which is poorly active with several cancer and normal DNA in vitro syntheses and DNA strand separation, is particularly active with breast cancer DNA (hormone target tissue DNA); this is true also for daunorubicin. Estradiol has a limited effect. It was reported that steroids are able to induce adenocarcinoma of the breast [19], to stimulate the growth of breast cancer [15, 23] and to induce erythroid differentiation in leukemia cells [14]. In patients receiving androgens, an increase of polynuclears or hematotoxicity was observed in the presence of those

drugs [31]. These results, and the results which show the additive effects of the above compounds on human neurocarcinoma DNA strand separation, suggest that all cancer DNA must possess some particular sites different for various DNA. The high susceptibility of cancer DNA to the compounds tested indicates that the double helical structure of cancer DNA might be destabilized, for instance by methylated bases whose level could be higher in cancer than in normal DNA. This view is compatible with the observation [13] that the presence of methylated adenine residue in DNA destabilizes the double helices in proportion to the frequency of occurrence of these residues. On the other hand, molecules such as peptides or amino acids bound in a different concentration to cancer and normal DNA, may participate in the stabilization and/or destabilization of the structure of a given DNA.

Of particular interest are the observations made with croton oil [source of phorbol derivatives; 16] and DMSO which, at low concentrations, are capable of enhancing DNA in vitro synthesis and strand separation not only of cancer DNA but also of normal DNA. When DNA strand separation is moderately induced by low doses of croton oil, the addition of DMBA further increases this process. It was demonstrated that the phorbol esters (isolated from croton oil), at high concentrations and in the absence of any other carcinogen, may lead to a low cancer incidence [16] and that the burst of DNA synthesis induced in skin by croton oil is preceded by a smaller and shorter stimulation of RNA and protein synthesis [17], a fact which should be related to the appearance of cancer cells in animals [6, 24]: it has been reported that croton oil is required as a promoter in the carcinogenesis initiated by a carcinogen [25]. Both croton oil derivatives and DMSO are capable of inducing differentiation of some cancer cell lines [14, 18]. Of particular interest is the observation that in human leukemic HL 60 cells, phorbol derivatives, at low concentrations, induced differentiation to macrophages whereas actinomycin D, which is carcinogenic in vitro [2] as well as in vivo [27], induced differentiation to granulocytes [21]. This drug is also capable of inducing neurite formation in mouse neuroblastoma cells [1].

Our in vitro observations on DNA synthesis and strand separation correlate with the multiplication of cancer cells. In fact, DMBA or CCNU, at relatively low concentrations, markedly stimulate the multiplication of Ehrlich ascites tumor cells in mice, while daunorubicin, cyclophosphamide or DMBA stimulate the multiplication of Crown gall tumor cells in plants, only when used at low concentrations: at higher concentrations, they inhibit tumor development [20]. These results might be connected with observations obtained in clinical studies for cancer treatment with chemotherapeutic agents. In order to kill cancer cells, sequential use of different drugs is commonly advised in therapy. It is conceivable that different drugs contribute to separate the strands of cancer DNA above a certain threshold (as we showed in vitro; fig. 7); once this is accomplished, the pathways for synthesis are disconnected and the cell is expected to die.

On the other hand, the relative resistance of DNA from healthy tissues to undergo in vitro DNA strand separation in the presence of carcinogens or other compounds might be connected with the fact that the effect of carcinogens never leads to instant cancer. It was reported that induction of cancer cells probably needs persistent gene activation and that the appearance of cancer cells is an additive

and cumulative process [7, 19]. *It is conceivable that carcinogens and carcinogen-like substances bind to some particular DNA sequences when used at low concentrations and might persistently tend to locally separate the double helical structure of normal DNA cells, thus permitting transcription of DNA into RNA up to a threshold which permits cell growth with new phenotypic expression.* This threshold may correspond to a 'switch' [30] allowing a cell to be modified and survive. Beyond this point, complete DNA strand separation may occur and thus lead to cell death.

On the basis of the results reported and discussed here, showing that chemically unrelated compounds do induce, to various extents, in vitro DNA strand separation and promote differentiation of particular cancer cells, it is conceivable that DNA strand separation is an obligatory step in the process required for the activation and expression of the genes. It strengthens the concept [9] that various molecules present in the cytoplasm of a cell may participate in the establishment of the cell programs, as recently suggested in the principles of automation during animal development.

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Received: June 10, 1980
Accepted: October 15, 1980

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