visualized is shown in Fig. 1C, as determined by a thallium-201 scan. Three animals that received the ^{111}In-DTPA-Fab intravenously were imaged 24 hours later; the infarct was clearly revealed, as shown for one of the dogs in Fig. 2, A and B. In each of the animals studied, the infarcts were identified postmortem by histochemical staining with triphenyltetrazolium chloride (TTC) (*8*). The stained region corresponded to the region of radioactive antibody concentration. A representative comparison is shown in Fig. 2, C and D. In three control animals, subjected to sham operation but not coronary ligation, there was no localization of labeled antibody either in vivo or in vitro.

In preliminary experiments, gamma camera images have also been obtained with technetium-99m–labeled antimyosin-Fab and positron camera images with gallium-68–labeled antimyosin Fab. Figure 3 shows a left lateral gamma image of ^{99m}Tc-DTPA-Fab localization in the anterolateral wall of the canine left ventricle, the site of experimental myocardial infarction after intravenous injection.

These experiments not only demonstrate the potential utility of specific antibody fragments in the localization of lesions characterized by unique antigenic identity in vivo, but also provide an approach to the labeling of such specific antibodies with various radionuclides that possess optimal imaging properties.

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22 October 1979; revised 19 December 1979

Mitochondrial DNA Is a Major Cellular Target for a Dihydrodiol-Epoxide Derivative of Benzo[*a*]pyrene

Abstract. *When mammalian cell cultures are exposed for 2 hours to (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo*[a]*pyrene, a mutagenic and carcinogenic derivative of benzo*[a]*pyrene, the extent of covalent modification of mitochondrial DNA is 40 to 90 times greater than that of nuclear DNA. Evidence is presented that this reflects the lipophilic character of the derivative and the very high ratio of lipid to DNA in mitochondria. These results suggest that mitochondrial DNA may be an important cellular target of chemical carcinogens.*

Recent studies have provided evidence that a dihydrodiol-epoxide, (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (BDPE), is the most potent carcinogenic and mutagenic metabolite formed in mammalian cells and tissues exposed to the environmental pollutant benzo[*a*]pyrene (*1*). The BPDE binds covalently to nuclear DNA (nDNA) and cellular RNA in vivo and also forms covalent adducts with a variety of nucleic acids in vitro (*1*). The major adduct consists of a guanine residue linked via its 2-amino group to the 10 position of benzo[*a*]pyrene (*1*). The covalent binding of BPDE or other ultimate carcinogens with nDNA is often emphasized as the initial and crucial event in the malignant transformation of cells by chemical carcinogens. We now report that in mammalian cell cultures exposed to BPDE, mitochondrial DNA (mtDNA) is modified to a much greater extent than nDNA. Our results raise the possibility that carcinogen modification of mtDNA may play an important role in the carcinogenic process.

When three different cell types were exposed to [^{3}H]BPDE for 2 hours, the extent of modification of mtDNA was much higher than that of nDNA (Table 1). The values of 3 to 12 BPDE residues per 10^6 DNA base residues that we ob-

Table 1. Extent of modification of nuclear DNA (nDNA) and mitochondrial DNA (mtDNA) when cell cultures are exposed to [^{3}H]BPDE. The 10T^1/$_2$ mouse embryo cell line (*12*) and a chloramphenicol-resistant subline of mouse L cells (*12*) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 percent fetal bovine serum (FBS) in 10-cm tissue culture plates (Nunclon) at 37°C in an atmosphere of 5 percent CO_2 and air. The rat liver epithelial cell line K-16 (*12*) was grown under the same conditions but in F12 medium with 10 percent FBS. The cellular DNA was uniformly labeled by growing the cells continuously in the presence of [^{14}C]thymidine (0.02 μCi/ml; 41 mCi/mmole) for five to six passages. Confluent cultures were washed with phosphate-buffered saline (PBS) and incubated at 37°C for 2 hours with 3 ml of serum-free medium per plate containing [^{3}H]BPDE (1.8 μg/ml; 411 mCi/mmole). Cells from 20 to 30 plates were then washed with PBS, dissociated with 0.1 percent trypsin-Versene and sedimented at 2500 rev/min. The cells were then resuspended in 5 ml of 0.25*M* sucrose containing 10 m*M* EDTA, bovine serum albumin (0.5 mg/ml), and 20 m*M* Hepes buffer, and homogenized in a glass homogenizer. The homogenate was centrifuged at 2500 rev/min for 5 minutes; the pellet was suspended in 3 ml of sucrose-buffer solution and recentrifuged to yield the nuclear pellet. Both supernatant fractions were combined and recentrifuged several times at 2500 rev/min until no visible pellet was seen. The final supernatant fraction was then centrifuged at 10,000 rev/min for 20 minutes to yield a mitochondrial pellet. To extract DNA, the nuclear and mitochondrial pellets were suspended for 1 hour at 37°C in a buffer solution containing 20 m*M* NaCl, 1 m*M* EDTA, 20 m*M* tris-HCl (*p*H 7.8), 0.1 percent sodium dodecyl sulfate, and proteinase K (200 μg/ml). The nDNA was then extracted with hot phenol and incubated for 1 hour with RNAase and then for 1 hour with proteinase K. The hot phenol extraction was repeated, and the nDNA was collected (*13*). To remove noncovalently bound radioactivity, the nDNA was solubilized in buffer containing 20 m*M* NaCl, 1 m*M* EDTA, 20 m*M* tris-HCl (*p*H 7.8), then sonicated, extracted several times with ethyl acetate until it reached a constant specific activity, precipitated with ethanol, and assayed for ^{14}C and ^{3}H content and absorbance at 260 nm. The resulting values were used to calculate extent of modification by [^{3}H]BPDE. The mtDNA was extracted several times with chloroform-isoamyl alcohol (24:1). The aqueous phase was then centrifuged in a CsCl-ethidium bromide gradient at 40,000 rev/min for 40 hours. A single peak of DNA banding at a density of 1.59 to 1.60 g/cm^3, corresponding to the known density of mammalian closed circular supercoiled mtDNA (*14*), was obtained. Fractions constituting this peak were combined, the ethidium bromide was extracted with isopropyl alcohol saturated with H_2O and CsCl, and any residual noncovalently bound [^{3}H]BPDE was removed by repeated extractions with ethyl acetate until the mtDNA had a constant specific activity. The mtDNA was then dialyzed overnight against buffer containing 2 m*M* NaCl, 0.1 m*M* EDTA, 2 m*M* tris-HCl (*p*H 7.8) and lyophilized. The extent to which mtDNA was modified by [^{3}H]BPDE was also calculated from the ratio of ^{3}H to ^{13}C.

Cell culture	Residues, [^{3}H]BPDE bound per 10^6 bases		Ratio of mtDNA to nDNA
	nDNA (No.)	mtDNA (No.)	
10T^1/$_2$	12	766	84
L cells	3	126	42
K-16	10	900	90

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tained for nDNA are comparable to previously published data for nDNA (*2*); the values that we obtained for mtDNA are about 40 to 90 times greater (Table 1). Since both the mtDNA and the nDNA were extensively extracted with organic solvents until they had constant specific activities, the results must be due to the covalently bound material and not to contamination by free [^{3}H]BPDE or degradation products. In addition, since the purified mtDNAs banded as sharp peaks in CsCl-ethidium bromide gradients at the density characteristic of mammalian closed circular supercoiled mtDNA (1.59 to 1.60 g/cm^3), they appear to be free from protein or nucleic acid contaminants. When similarly analyzed, the nDNA banded at its characteristic density (1.52 to 1.54 g/cm^3). In the experiments presented in Table 1, the amounts of mtDNA were determined by prelabeling the cells with [^{14}C]thymidine, since for routine experiments it was difficult to obtain sufficient amounts of unlabeled mtDNA for quantitation. Separate experiments with larger amounts of cells and quantitation of both mtDNA and nDNA by absorbance at 260 nm indicated that the [^{14}C]thymidine labeling method was valid.

The above results cannot be attributed to chemical or conformational properties peculiar to mtDNA. When purified samples of mtDNA and nDNA were reacted under similar conditions with [^{3}H]BPDE in aqueous solution, the extent of in vitro modification for both mtDNA and nDNA was approximately three [^{3}H]BPDE residues per 1000 bases. This value is in the same range as that obtained in previous modification studies in which either linear calf thymus DNA or supercoiled plasmid DNA was used as substrate (*3*). During these in vitro studies, we found that the addition of $2 \times 10^{-2} M$ Mg^{2+} to the reaction mixture resulted in a three- to fourfold inhibition of DNA modification for both mtDNA and nDNA, presumably because a more rigid DNA helix is less reactive with BPDE. Thus the high extent of modification of mtDNA in vivo is not simply due to greater susceptibility of supercoiled closed circular DNA to chemical modification. Nor is it likely that the much higher modification of mtDNA by BPDE in vivo is due to protection of nDNA from chemical modification by the chromatin structure, since previous results indicate that BPDE modification of purified DNA is only two to three times that of DNA in chromatin core particles (*4*).

To evaluate the possible role of DNA repair, we incubated confluent cultures of 10T^1/$_2$ with [^{3}H]thymidine during a 2-hour incubation with [^{14}C]BPDE. The incorporation of [^{3}H]thymidine into the nDNA of the BPDE-treated cells was not significantly increased over that of control cultures not exposed to BPDE, evidence that during this initial 2-hour period there is negligible excision repair. This result is consistent with our previous results, which indicated that the excision of BPDE adducts from nDNA proceeds relatively slowly; it takes at least 68 hours to remove 70 percent of the BPDE adducts from the nDNA of 10T^1/$_2$ cells (*2*). It is thus extremely unlikely that nDNA has fewer BPDE adducts than mtDNA (Table 1) because of an extremely rapid and efficient nuclear DNA excision and repair process. Although relatively little is known regarding DNA repair in mitochondria (*5*), this subject takes on added significance in view of the present results indicating extensive carcinogen modification of mtDNA.

Table 2. Intracellular distribution of [^{14}C-]-BPDE in 10T^1/$_2$ cells. Confluent 10T^1/$_2$ cell cultures were exposed to [^{14}C]BPDE (0.5 μg/ml; 27 mCi/mmole) for 20 minutes, at which time ~ 50 percent of the radioactivity was associated with cells. The cells were immediately scraped with a rubber policeman and lysed with 0.5 percent NP-40. Nuclei were obtained by centrifugation at 2500 rev/min for 5 minutes and mitochondria by centrifugation at 10,000 rev/min for 20 minutes. The mitochondrial supernatant fraction includes membrane fragments, microsomes, and cytosol, and was not further fractionated.

Cell fraction	Percent of total cell-associated [^{14}C]BPDE
Nucleus	30
Mitochondria	15
Mitochondrial supernatant	55

Another approach is to consider the intracellular distribution of BPDE. When confluent 10T^1/$_2$ cultures were incubated with [^{3}H]BPDE or [^{4}C]BPDE for 2 hours, 30 to 50 percent of the radioactivity added to the medium was associated with the cells. The nuclear and mitochondrial fractions contained 30 and 15 percent, respectively, of the total cell-associated [^{14}C]BPDE (Table 2). Previous studies (*6*) indicated that polycyclic aromatic hydrocarbons also accumulate in mitochondria. In agreement with previous results for mammalian cells (*7*), we found that the ratio of total nDNA to mtDNA was approximately 100:1. Thus, the ratio of total BPDE to DNA may be about 50 times greater in mitochondria than that in the nucleus. Presumably, the relatively high uptake of BPDE by mitochondria reflects the high membrane lipid content of mitochondria (*8*) and the highly lipophilic character of BPDE. These factors may explain the extensive modification of mtDNA in cells exposed to [^{3}H]BPDE (Table 1).

To determine whether BPDE present in a lipid phase can react covalently with DNA, we reacted [^{3}H]BPDE with DNA when both were attached to sonicated phosphatidylcholine-cholesterol liposomes (PCL's). In the presence of Mg^{2+} or Ca^{2+} ions, nucleic acids bind to the external surface of PCL's; they also bind to liposomes prepared from total mitochondrial lipid and to the external surface of mitochondria or mitoplasts (*9*). After PCL's were incubated with purified nDNA and [^{3}H]BPDE in the presence of Mg^{2+} at 37°C for 5 minutes, the amount of radioactivity covalently bound to the repurified DNA was determined (Table 3).

Table 3. Effect of DNA binding to liposomes on [^{3}H]BPDE modification. Sonicated phosphatidylcholine-cholesterol liposomes at a molar ratio of 3:1 were prepared as described (*8*) and incubated at 7.5 mg per milliliter of lipid with 18 μM ^{14}C-labeled nDNA and the indicated concentration of [^{3}H]BPDE (466 mCi/mmole) for 5 minutes at 37°C in $2 \times 10^{-2} M$ Mg^{2+} in buffer containing 20 mM NaCl, 1 mM EDTA, and 20 mM tris-HCl (pH 7.8). Parallel assays were run under the same conditions but in the absence of liposomes. Portions were solubilized with 0.1 percent sodium dodecyl sulfate and repeatedly extracted with ethyl acetate to remove noncovalently bound material. The DNA was precipitated with ethanol and assayed for [^{3}H]BPDE binding. Portions of the reaction mixture were also directly centrifuged at 40,000 rev/min for 3 hours, and the total amounts of DNA and [^{3}H]BPDE associated with the liposome pellet were determined. The latter values were 90 and 95 percent, respectively, of the initially added amounts.

[^{3}H]BPDE concentration	Residues, [^{3}H]BPDE bound per 10^6 bases	
	Number with liposomes	Number without liposomes
85 nM	160	180
250 nM	2100	2800

Portions from the same reaction mixture were centrifuged at 40,000 rev/min, and the total amounts of [^{3}H]BPDE and DNA associated with PCL's were determined. Under these conditions, about 90 percent of the initially added DNA and 95 percent of the [^{3}H]BPDE were found in the sedimented liposomes. The extent of DNA modification by [^{3}H]BPDE when the DNA was associated with PCL's was approximately the same as that found when [^{3}H]BPDE was reacted with free DNA in the absence of PCL's (Table 3). Thus neither the solubilization of [^{3}H]BPDE in a lipid phase nor the attachment of DNA to lipids interferes

with the covalent modification of the DNA. In fact, we have found that solubilization of BPDE in a lipid phase enhances DNA modification by stabilizing the BPDE (*10*).

Taken together, our results suggest that when cells are exposed to [^{3}H]BPDE, the high uptake of the compound by mitochondrial membranes and the high lipid-to-DNA ratio of the mitochondria result in extensive modification of mtDNA by this carcinogen. Studies in progress indicate that when 10T$^1/_2$ cells are incubated with the parent hydrocarbon benzo[*a*]pyrene, there is also much more extensive modification of mtDNA than of nDNA (*10*). It is of interest that Wunderlich *et al.* (*11*) found that when *N*-nitroso-*N*-methylurea or *N*-nitrosodimethylamine was administered to rats there was preferential methylation of liver mitochondrial DNA; in vitro studies by these investigators led them to conclude that their results reflected concentration of the lipophilic carcinogens in the mitochondria, rather than preferential susceptibility of mitochondrial DNA to chemical modification. Further studies are required to assess the functional consequences of preferential modification of mtDNA by chemical carcinogens and the possible relevance of this effect to the carcinogenic process.

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15. This research was supported by National Cancer Institute grant CA 21111. We thank the Chemical Repository of NCI for providing the [^{3}H]BPDE and [^{14}C]BPDE used in these studies.

25 February 1980

Specific Locus Mutations Induced in Somatic Cells of Rats by Orally and Parenterally Administered Procarbazine

Abstract. *A new test, the granuloma pouch assay, was used in detecting specific locus mutations in somatic cells of rats in vivo after the animals were treated orally and parenterally with procarbazine hydrochloride, an agent used in cancer chemotherapy. The results indicate that stable intermediates are formed in the body and distributed as proximate mutagens.*

Mutagenicity tests are widely accepted as a quick means for detecting carcinogens. However, the assessment of mutagenic events in somatic cells of intact, adult laboratory animals is restricted to clastogenic effects and sister chromatid exchanges in dividing cell populations such as bone marrow, germ cells, and lymphocytes stimulated in vitro. Presently available specific locus assays in mammalian cells are based on clone formation of mutant cells in selective media. In vivo, these assays are impeded by the low proliferative activity of cells in most target organs and, in vitro, by their limited cloning efficiency.

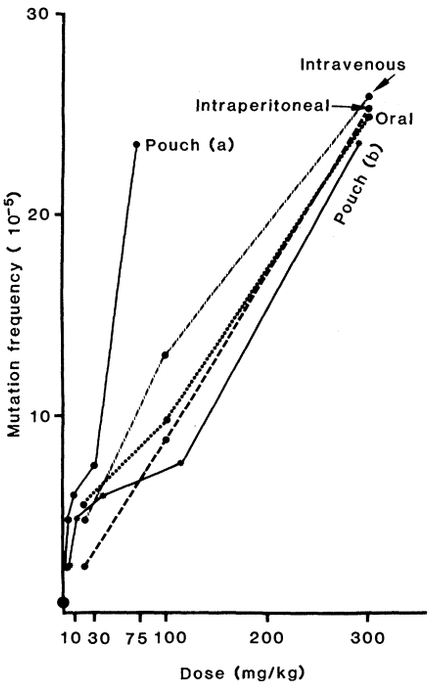

Fig. 1. Procarbazine dose-response curves for different routes of administration. Curves for the five doses injected directly into the pouch are plotted twice, as milligrams per pouch (a) and as the equivalent in milligrams per kilogram of body weight (4.0, 11.6, 38.6, 117.6, and 289.2 mg/kg) (b). The mutation frequency of control animals was 0.53×10^{-5}.

The mutagenicity of *N*-isopropyl-α-(2-methylhydrazino)-*p*-toluamide HCl (procarbazine HCl), widely used in tumor chemotherapy, was investigated in vivo with a new test, the granuloma pouch assay (*1*, *2*). With this system, chemically induced specific locus mutations can be detected in somatic cells of intact adult rats. Procarbazine is carcinogenic in rodents (*3*) and primates (*4*) and may induce secondary tumors in human patients (*5*).

Growth of granulation tissue was initiated in rats at the inside of a subcutaneous air pouch by injecting a small amount of croton oil. Forty-eight hours later, freshly prepared procarbazine solution in 0.9 percent NaCl was administered systemically (intravenously, orally, and intraperitoneally) in doses of 20, 100, and 300 mg/kg, or was injected directly into the pouch (1, 3, 10, 30, and 75 mg). Control animals were treated with 0.9 percent NaCl alone, injected directly into the pouch. The animals were killed after 48 hours, and the granulation tissue was dissected and dissociated enzymatically into single cells. Mutation frequencies were then determined in medium containing 10 μ*M* 6-thioguanine (*1*, *2*).

The results demonstrate that procarbazine induces specific locus muta-

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