tion by epinephrine were significantly lower than those for ADP, showing some selectivity of their effect on the response to epinephrine. The unidentate binding agent 3-chloropyridine and 4,4′-dipyridyl, which has two liganding sites but is not a classical bidentate chelator, were effective in concentrations only slightly different from the bidentate chelator 2,2′-dipyridyl. Since the heme prosthetic group allows liganding to only one site on the iron by one molecule of these inhibitors (*12*), a unidentate iron-binding agent like 3-chloropyridine should be active, as indeed it was, in concentrations similar to those of 2,2′-dipyridyl. The results are therefore consistent with the concept that heme reduction is involved in the action of epinephrine on platelets.

The nature of the platelet process that might be activated by heme reduction is not known. One early event in the platelet response to epinephrine is inhibition of adenylate cyclase, but this process has been dissociated from stimulation of aggregation (*13*). Further study of the potentially important and perhaps widespread role of heme reduction in signal transmission will require better definition of this initial event.

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4. Heme was prepared in a 25 percent alkaline pyridine solution, *p*H 12.6, and diluted in 30 percent pyridine, *p*H 12.2, to a concentration of 0.77 or $1.54 \times 10^{-4} M$ in an ultraviolet cuvette. Heme reduction was then followed by monitoring absorption at 558 nm and quantified by $E = 30\ mM^{-1}$, where E is the extinction coefficient as described by D. A. Peterson, J. M. Gerrard, G. H. R. Rao, and J. G. White [*Prostaglandins Med.* **4**, 73 (1980)].
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14. We are grateful for financial support for these studies from USPHS grant HL 11880, grant MA7396 from the Canadian MRC, and a grant from the Children's Hospital of Winnipeg Research Foundation. J.M.G. is the recipient of a Canadian MRC scholarship. Send reprint requests and correspondence to J.M.G.

18 March 1981; revised 17 July 1981

Preferential Attack of Mitochondrial DNA by Aflatoxin B_1 During Hepatocarcinogenesis

Abstract. *Administration of the hepatic carcinogen aflatoxin B_1 to experimental animals results in covalent binding to liver mitochondrial DNA at concentrations three to four times higher than nuclear DNA. The concentration of carcinogen adducts in mitochondrial DNA remains unchanged even after 24 hours, possibly because of lack of excision repair. Similarly, mitochondrial transcription and translation remain inhibited up to 24 hours suggesting long-term effects of aflatoxin B_1 on the mitochondrial genetic system.*

Mitochondria from tumor cells have altered functional and structural properties (*1*). It is not known if these abnormalities are due to altered mitochondrial genetic systems or indirect effects involving the nuclear genes. Recent studies in our laboratory showed the presence of a cytochrome P-450 type of monooxygenase system in rat liver mitochondria capable of activating the hepatic carcinogen aflatoxin B_1 (AFB_1) into an electrophilic reactive form which then covalently modifies mitochondrial DNA, RNA, and protein (*2*). Activated metabolites of benzo[*a*]pyrene and other polycyclic aromatic hydrocarbons (*3*) incubated with tissue culture cells cause substantially more modification of mitochondrial DNA than of nuclear DNA. We now report that AFB_1 administered to experimental animals covalently binds to liver mitochondrial DNA more than to nuclear DNA. The chemical modification of mitochondrial DNA persists even after 24 hours and is accompanied by pronounced inhibition of mitochondrial RNA and protein synthetic activities.

When AFB_1 is administered intraperitoneally to rats, the amount of carcinogen in the hepatic tissue increases rapidly to reach a peak in about 2 to 3 hours with 15 to 18 percent of the drug dose localized in this tissue (*4*). At this time, nearly 60 percent of hepatic AFB_1 is in covalently bound form. Table 1 shows that at a peak time of 3 hours almost 24 percent of the bound AFB_1 is associated with mitochondria whereas 22 percent is

Table 1. Intracellular distribution of AFB_1 in hepatic tissue. Male Sprague-Dawley rats (150 to 180 g) were injected intraperitoneally with 30 μCi of [^{3}H]AFB_1 (30 μCi/2.9 μmole) to yield a dose rate of 6 mg/kg. At intervals, the rats were killed and the livers were removed, washed free of blood clots, minced, and homogenized in 2 m*M* Hepes (*p*H 7.5), 70 m*M* sucrose, 220 m*M* D-mannitol, 1 m*M* EDTA, and 0.5 g of bovine serum albumin per liter as described (*2*). The homogenate was adjusted to 10 percent (weight to volume) with the above buffer and used for isolating the nuclear fraction (the pellet obtained at 1100 *g*), mitochondrial fraction (the pellet obtained at 8500*g*), and postmitochondrial supernatant as described (*2, 10*). The nuclear pellet was washed twice with buffer containing 20 m*M* tris-HCl (*p*H 7.5), 60 m*M* KCl, 2 m*M* $CaCl_2$, and 0.5 percent Triton X-100 to remove contaminating membranes (*12*); the mitochondrial pellet was washed with digitonin (0.1 mg per milligram of protein) to remove the outer membrane (*13*), and the soluble fractions from these washings were included in the postmitochondrial supernatant. The amount of bound [^{3}H]AFB_1 in each fraction was assayed by precipitation with cold CCl_3COOH as described (*2*). Data from three independent estimates were used for calculating the mean and standard deviations. Values in parentheses are percentages of the total cellular bound [^{3}H]AFB_1 at the specified time points.

Cell fraction	Amount of [^{3}H]AFB_1 bound (nmole)		
	At 3 hours	At 12 hours	At 24 hours
Total intracellular	199.0 ± 12.1 (100)	127.0 ± 4.2 (100)	56.0 ± 3.2 (100)
Post mitochondrial supernatant	109.0 ± 8.1 (54.4)	67.5 ± 2.1 (53.4)	22.9 ± 2.9 (38.2)
Mitoplasts	49.6 ± 4.1 (23.5)	36.5 ± 4.1 (28.7)	27.1 ± 4.8 (49.6)
Nucleus	44.1 ± 4.6 (22.1)	20.9 ± 2.2 (15.0)	6.4 ± 0.77 (12.1)

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in the nuclear fraction. If one considers that the mitochondrial fraction in liver corresponds to about 8 to 10 percent of the cell mass (*5*), it appears that the amount of bound AFB_1 in relation to mass in the mitochondrial fraction is four to six times greater than in any other cell compartment. Furthermore, the AFB_1 in the nuclear and postmitochondrial fractions declines sharply with time, in contrast to a surprisingly slower rate of removal from the mitochondrial fraction. Thus, 24 hours after administration, the mitochondrial fraction contains nearly 50 percent of the total intracellular bound AFB_1 whereas the nuclear fraction contains only 12 percent of the total.

Studies of the binding of AFB_1 to DNA (Fig. 1) show that under the same experimental conditions, mitochondrial DNA is modified preferentially when compared to nuclear DNA. At about 3 hours after AFB_1 administration, as many as 10 to 12 adducts per 10^7 daltons of mitochondrial circular DNA and only about 3 adducts per 10^7 daltons of nuclear DNA are detected. This preferential binding to mitochondrial DNA may be due to higher amounts of the lipophilic carcinogen AFB_1 being sequestered in these membranous organelles (*3*), although the less compact packaging of mitochondrial circular DNA (*6*) compared to the more compact chromosomal packaging cannot be ruled out. In agreement with the relative rates of AFB_1 removal from the various cell fractions, a linear decline in the number of adducts per unit of molecular size DNA occurs in the nuclear DNA. In contrast, the level of adducts in the mitochondrial DNA remains nearly constant even after 24 hours. The values on the binding of AFB_1 to mitochondrial and nuclear DNA (Fig. 1) are highly reproducible within about 8 percent of the variations between separate estimates.

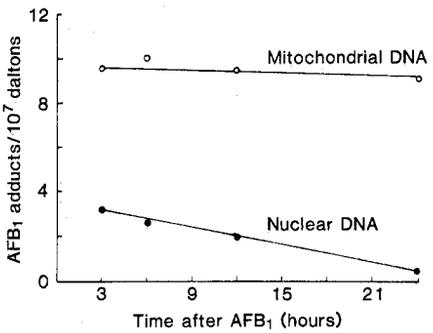

Fig. 1. Distinctive patterns of AFB_1 binding to nuclear and mitochondrial DNA in vivo. Rats were injected with [^{3}H]AFB_1 at a dose rate of 6 mg/kg (30 μCi/2.9 μmole). At intervals the rats were killed and their livers removed and used for isolating mitoplasts and nuclei as described in Table 1. The mitoplasts were lysed with 2 percent sodium dodecyl sulfate in a buffer containing 20 m*M* tris-HCl (*p*H 7.6), 100 m*M* NaCl, and 25 m*M* EDTA; the mitochondrial circular DNA was separated by density banding in cesium chloride–ethidium bromide gradients according to the method of Hudson *et al.* (*14*). Nuclear DNA was isolated by the phenol-cresol method (*15*) and was further purified by banding cesium chloride–ethidium bromide gradients as above. The mitochondrial DNA resolves into linear (upper) and circular (lower) bands whereas the nuclear DNA bands as a single linear component. The mitochondrial (circular) and nuclear DNA bands were removed with a syringe and freed of ethidium bromide and cesium chloride as described (*2*). The bound radioactivity in the DNA was determined by counting portions with 10 ml of Cab-o-Sil scintillation mixture as described (*2*). Values represent the mean of two independent experiments.

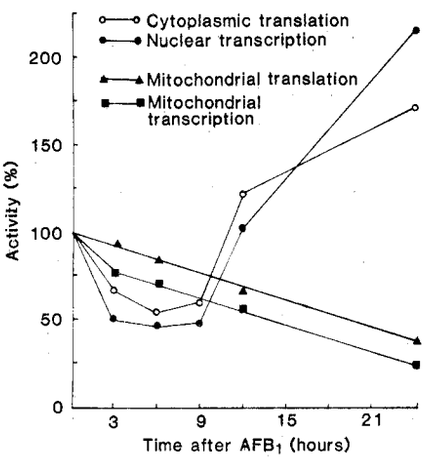

Fig. 2. Long-term inhibition of mitochondrial transcription and translation after the administration of a single dose of 6 mg of AFB_1 per kilogram. Intact hepatocytes prepared by the collagenase method (*9, 12*) were used to study cytoplasmic translation and nuclear transcription. Cells were labeled with [^{35}S]methionine (100 μCi/ml; 1100 Ci/mmole) or with [^{3}H]orotic acid (100 μCi/ml; 28 Ci/mmole) as described (*12*). The amount of [^{35}S]methionine (8.4×10^6 counts per minute per 10^6 cells) and [^{3}H]orotic acid (2.6×10^5 counts per minute per 10^6 cells) incorporated after 120 minutes of labeling control hepatocytes were considered 100 percent cytoplasmic translation and nuclear transcription activities, respectively. Isolated mitoplasts were used to study mitochondrial transcription and translation activities. Mitoplasts were labeled with [^{35}S]methionine (150 μCi/ml) or ^{3}H-labeled guanosine triphosphate and ^{3}H-labeled cytidine triphosphate (20 μCi/ml each) as described (*2*). The level of [^{35}S]methionine (0.86×10^6 counts per minute per milligram of protein and of [^{3}H]CTP and [^{3}H]GTP (0.32×10^5 counts per minute) incorporated by control mitoplasts after 60 minutes of incubation were regarded as 100 percent mitochondrial translation and transcription activities respectively. Values represent the mean of two independent experiments for each point presented.

The concentration of carcinogen and the time of retention are important factors directly related to the concentrations as well as types of adducts found on the DNA. In keeping with this, our results on the binding of AFB_1 to nuclear and mitochondrial DNA show contrasting features of the two systems. First, the binding of AFB_1 to mitochondrial DNA is three to four times higher than to nuclear DNA. This observation is consistent with previous reports of preferential binding of various carcinogenic methylating agents, including *N*-nitrosodimethylamine, to mitochondrial DNA in vivo (*7*) and of polycyclic aromatic hydrocarbons incubated with tissue culture cells (*3*). Second, the AFB_1 adducts on the mitochondrial circular DNA remained nearly constant even after 24 hours, possibly because of the lack of an appropriate excision system in these organelles (*8*).

The effect of AFB_1 modification of template DNA on the transcription and translation processes in the two genetic systems was determined by using intact hepatocytes (*9*) and isolated mitoplast systems (*10*). Both nuclear transcription and cytoplasmic translation are inhibited by 70 to 80 percent in about 3 to 6 hours after AFB_1 administration (Fig. 2). Both of these activities recover to control levels by about 12 hours and reach a peak of 160 to 220 percent by 24 hours, possibly reflecting the nuclear repair process. Mitochondrial transcription and translation activities, in contrast, remain inhibited even 24 hours after AFB_1 administration. In two separate experiments, the values on RNA and protein synthesis were reproducible within an error range of 10 percent. Although the results presented here reflect the activity in isolated mitoplasts, mitochondrial specific translation and transcription in intact hepatocytes in the presence of specific inhibitors show identical AFB_1 inhibitory patterns, suggesting that the time course of AFB_1 inhibition obtained with isolated mitoplasts reflects the actual status of these activities in vivo.

Thus our results show that mitochondria are the direct and possibly one of the major cellular targets for AFB_1 during experimental carcinogenesis. Preferential binding to mitochondrial DNA by AFB_1 and other carcinogens (*3, 7*), along with the results on induction of petite mutation in yeast (*11*) by various carcinogenic agents, shows that mitochondrial DNA is a critical target. This modifica-

tion of mitochondrial DNA leading to long-term inhibition of mitochondrial biosynthetic processes and perhaps to mitochondrial mutational events may directly contribute to the carcinogenic process.

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17 July 1981

Substance P Counteracts Neurotoxin Damage on Norepinephrine Neurons in Rat Brain During Ontogeny

Abstract. *Systemic treatment of newborn rats with the catecholamine neurotoxin 6-hydroxydopamine alters the postnatal development of the central norepinephrine neurons. The changes are permanent and consist of denervation of distant nerve terminal projections (for example, cerebral cortex) and hyperinnervation of terminal areas close to the cell bodies (for example, cerebellum). Intracisternal injection of substance P counteracted both of these alterations. The results indicate that substance P may prevent degeneration of damaged norepinephrine neurons during ontogeny or may have a regrowth stimulatory action on these cells. Substance P might prove of use in the prevention or reduction of other types of neurodegenerative disease.*

The monoamine-containing neurons in the central nervous system (CNS) appear very early during development in many species including man. In rat the cell bodies are already formed on gestation days 12 to 14, and the axonal pathways are fully developed at birth. The postnatal development of central monoamine neurons consists mainly of a proliferation of the nerve terminals in the regions that they innervate (*1*). Systemic administration of the catecholamine neurotoxin 6-hydroxydopamine (6-OHDA) in the neonatal stage markedly alters the postnatal development of the central norepinephrine (NE) neurons, in particular those of the locus coeruleus, which has extensive collaterals and innervates many brain regions. This treatment thus leads to permanent and pronounced denervations of distant NE terminal projections (for example, in the cerebral cortex and spinal cord), whereas projections close to the cell bodies (for example, in the cerebellum and pons medulla) increase, leading to a hyperinnervation (*2*, *3*).

The consequences of neonatal administration of 6-OHDA on the central NE neurons are mainly related to a "pruning effect" (*4*). The data point to a rigid growth pattern of these neurons, especially with respect to the development of the number of NE nerve terminals, independent of the regions they innervate. The development of axonal arborizations and terminals may therefore be regulated mainly at the level of the perikarya, for example, by way of their synaptic inputs. The NE perikarya of the locus coeruleus receive various types of inputs, among others, nerve terminals containing the peptide substance P (SP) (*5*), which has an excitatory effect (*6*). We now report that neonatal administration of SP can modify the altered postnatal development of central NE neurons induced by neonatal administration of 6-OHDA.

Newborn rats (Sprague-Dawley) were treated with 6-OHDA (100 mg/kg, subcutaneously) or substance P (2×5 to 50 μg in 10 μl of solvent, intracisternally, at intervals of 20 hours). Some animals received both 6-OHDA and SP, in which case the first dose of SP was administered 4 to 6 hours after the 6-OHDA. Controls received identical injections of the solvent. The rats were raised to the age of 3 to 6 weeks, when they were killed and samples were taken from the CNS for neurochemical and histochemical analyses. Endogenous NE was assayed according to the method of Keller *et al.* (*7*) and the uptake of ^{3}H-labeled NE was measured in vitro as described by Lidbrink and Jonsson (*8*). Fluorescence histochemical demonstration of catecholamines was performed according to the method of Lorén *et al.* (*9*).

In agreement with previous studies (*2*, *3*) and compatible with the pruning effect concept, the neonatal administration of 6-OHDA depleted endogenous NE in the frontal cortex (−90 percent), the occipital cortex (−99 percent), and the spinal cord (−97 percent), whereas this treatment more than doubled the NE concentrations in the cerebellum and the pons medulla (Table 1). Administration of SP (2×50 μg) alone did not significantly alter NE except in the pons medulla where a small (+16 percent) but significant ($P < .05$) increase was found. Intracisternal injection of SP (2×50 μg) in animals previously treated with 6-OHDA significantly counteracted the NE depleting effect induced by 6-OHDA. This effect of SP was most pronounced in the frontal cortex. Substance P also counteracted the 6-OHDA–induced increases of NE, both in the pons medulla and cerebellum.

We also tested the effect of various doses of SP on the altered NE concentrations produced by 6-OHDA in the frontal cortex. The results demonstrated a clear dose-response relationship; 2×5 μg of SP had no effect (17 ± 9.4 percent of control) on the NE reduction produced by 6-OHDA (15 ± 4.1 percent of control), whereas both 2×10 μg and 2×25 μg of SP had clear counteracting effects, the NE levels being 35 ± 9.6 and 54 ± 6.3 percent, respectively, of control (191 ± 24 ng/g; $N = 4$ and 5, respectively). The effectiveness of 2×25 μg of SP was approximately similar to that of 2×50 μg (see Table 1).

In agreement with the results of the NE assay, 2×50 μg of SP had a normal-

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