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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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.

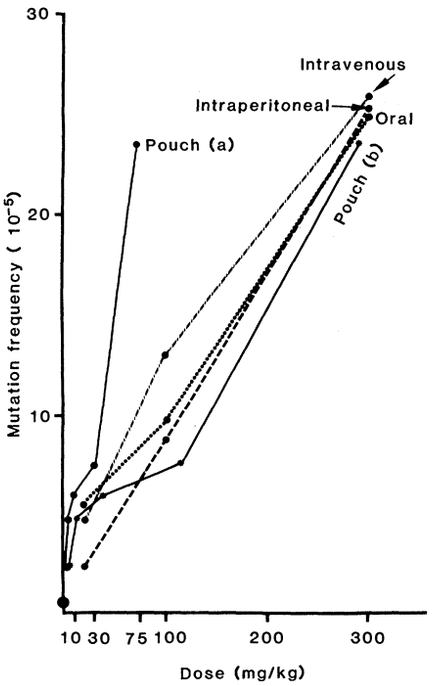

Fig. 1. Procarbazine dose-response curves for different routes of administration. Curves for the five doses injected directly into the pouch are plotted twice, as milligrams per pouch (a) and as the equivalent in milligrams per kilogram of body weight (4.0, 11.6, 38.6, 117.6, and 289.2 mg/kg) (b). The 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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Table 1. Procarbazine-induced mutation frequencies. The primary cloning efficiency was determined from cells recovered after enzymatic dissociation of the granulation tissue. The cells were cultured in air (8 percent CO_2, 95 percent relative humidity, 37°C) for 8 days in Dulbecco's minimum essential medium (DMEM) supplemented with 10 percent fetal calf serum. The secondary cloning efficiency was determined from cells dispersed with trypsin after a 3-day expression period. These cells were cultured for 8 days in DMEM with 10 percent dialyzed fetal calf serum. Mutant cells that were able to form clones were selected after being cultured for 11 days in 10 percent dialyzed fetal calf serum containing 10 μM 6-thioguanine. Mutation frequencies were calculated by determining the number of resistant cells per 10^5 cells able to form a clone (secondary cloning efficiency). Mutation frequencies from treated groups of animals are significantly different from control values at $P < .01$ (*21*).

Administration route	Dose		Number of animals tested	Mean cloning efficiency (%)		Number of clone-forming cells tested $\times 10^5$	Mutation frequency $\times 10^{-5}$	
	Milligrams per kilogram	Milligrams per pouch		Primary	Secondary		Mean ± standard deviation	Range
Oral	20.0		3	8.63	15.65	9.39	5.54 ± 7.07	1.29 to 33.70
	100.0		4	5.95	8.00	3.84	9.72 ± 9.04	2.94 to 22.45
	300.0		4	6.18	18.40	9.20	25.17 ± 8.51	15.98 to 34.20
Intravenous	20.0		3	9.93	21.30	9.60	4.77 ± 4.39	1.73 to 9.80
	100.0		4	4.88	36.50	15.35	13.12 ± 11.18	4.78 to 29.20
	300.0		5	7.54	21.70	22.11	26.10 ± 23.35	4.39 to 60.80
Intraperitoneal	20.0		3	5.78	8.00	3.07	2.37 ± 0.98	1.77 to 3.50
	100.0		3	7.67	17.10	5.81	8.81 ± 4.02	5.30 to 13.20
	300.0		3	7.00	26.10	6.11	25.30 ± 19.94	10.36 to 47.95
Pouch		1.0	2	4.90	34.30	9.47	2.35 ± 2.66	0.47 to 4.23
		3.0	3	8.70	29.90	13.46	4.76 ± 3.46	0.77 to 6.88
		10.0	3	4.69	18.70	6.99	6.07 ± 4.21	1.22 to 8.76
		30.0	4	5.81	17.5	10.34	7.53 ± 3.00	3.17 to 10.00
		75.0	3	6.00	19.30	6.42	23.63 ± 14.53	13.77 to 40.32
Controls (0.9 percent NaCl)			6	7.8	31.30	5.48	0.53 ± 0.51	0 to 1.40

tions in somatic extrahepatic cells in vivo (Table 1). There was a linear dose-effect relation for all administration routes. The dose-response curves obtained after intravenous, intraperitoneal, and oral administration were nearly identical (Fig. 1). These observations indicate that procarbazine is well absorbed at all administration sites and is distributed evenly throughout the body. When the dose administered locally into the pouch was converted to its equivalent in milligrams per kilogram, we obtained a dose-response relation similar to that seen with the other administration routes (Fig. 1). This indicates that procarbazine was not transformed locally to a mutagenic metabolite by granulation tissue cells. In liver homogenates, three pathways are postulated to lead to inactivation and the formation of alkylating intermediates and free radicals (*6*), whereas the formation of the proximate mutagen cannot be achieved in vitro with the hepatic microsomal enzyme preparations used in bacterial mutagenicity tests (*7*).

In vivo, according to our results, it is likely that stable, still unidentified procarbazine intermediates are formed in a central organ (probably the liver). After release into the circulation, these metabolites reach the granulation tissue, which is well supplied with blood. The final reactive species, interacting with DNA, might then be formed in a second intracellular activation-degradation step.

The present findings are in good agreement with published data showing genotoxic and carcinogenic effects of procarbazine in other target organs: The compound was clastogenic in germ cells (*8, 9*) and somatic cells (*10*), and it induced specific locus mutations in mice spermatogonia (*11*) and unscheduled DNA synthesis in rabbit germ cells (*12*). Procarbazine also caused subcutaneous tumors in rats after a single oral or intraperitoneal administration, and the incidence was comparable for both routes (*13*). The presence of this activity in several different extrahepatic tissues also supports the hypothesis that stable intermediates are distributed throughout the body, and even cross the blood-testes barrier. In contrast, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) was found to induce specific locus mutations only when injected into the granuloma pouch. The compound was not active when given orally or intraperitoneally (*1*). After MNNG was given orally, carcinomas of the stomach were found (*14*), and after repeated subcutaneous injections (*15*) or a single injection into the pouch (*16*), fibrosarcomas at the administration site were found. This indicates that MNNG has only an immediate action at the site of injection and explains the lack of mutagenic activity in germ cells (*17*) and bone marrow cells (*18*).

Our results illustrate the usefulness of the granuloma pouch assay in testing chemicals for mutagenicity and carcinogenicity. Within approximately 3 weeks, the procedure provides a qualitative and quantitative assessment of mutagenic activity. A further advantage is that the target tissue is exposed in animals whose pharmacokinetic and metabolic functions are intact. The comparison of the mutagenic activities after systemic and local administration of the agent can therefore provide important indications about the formation and distribution of the proximate mutagen.

The same target tissue can also be used for demonstrating breakage of DNA strands (*19*), sister chromatid exchanges, chromosome aberrations (*20*) and in situ tumor development (*16*). With this combined approach, it is possible to draw a correlation between the mutagenic and carcinogenic effects of chemicals.

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Age-Related Changes in Passive Avoidance Retention: Modulation with Dietary Choline

Abstract. *Two studies were performed to evaluate the effects of dietary choline manipulation on behavior in mice. Retired breeder mice (8.5 months old) were placed on purified diets that were either deficient in free choline or choline-enriched. After 4.5 months, the mice were trained in a single-trial, passive avoidance task and tested for retention either 24 hours or 5 days later. Their performance was compared with that of mice, of various ages, that were maintained on a control diet. The two salient findings were (i) a dramatic decrease in retention of the task in senescent mice (23 months and older) and (ii) marked behavioral differences between choline-deficient and choline-enriched mice (13 months old). In fact, the choline-enriched mice performed as well as 3-month-old mice, whereas the choline-deficient mice performed as poorly as the senescent mice. In a replication, three groups of retired breeder mice were placed on the same choline-deficient diet; control and enriched groups were given choline through their drinking water. Again, retention of learning was superior in the choline-enriched mice and inferior in the choline-deficient mice. These studies demonstrate that dietary manipulation of choline can significantly alter behavior in ways that are qualitatively and quantitatively similar to those occurring across the life-span of the mouse. Thus certain behavioral changes that occur with age might be modulated through appropriate precursor control.*

Evidence has accumulated that cholinergic dysfunctions may play a particularly important role in the memory impairments that occur with old age. This evidence, involving psychopharmacological (*1–4*), biochemical (*5*), and electrophysiological (*6*) studies of humans and infrahumans, has stimulated interest in the possibility that these age-related impairments might be reduced by pharmacological manipulation of the cholinergic system. Despite the compelling empirical support for this approach, attempts to pharmacologically modify the age-related impairments have thus far proven therapeutically disappointing (*7*).

It has been suggested that dietary manipulation of precursors to the cholinergic system may provide an alternative method of enhancing presynaptic cholinergic activity (*8*). Numerous studies have demonstrated that systemic or dietary manipulation of choline, the precursor for synthesis of acetylcholine, increases central cholinergic activity. (Manipulation of lecithin, the normal dietary source of choline, has the same effect.) In addition to the somewhat controversial findings that acetylcholine levels are altered by precursor manipulation (*8, 9*), significant changes in the activity of the synthesizing enzyme choline acetyltransferase (*10*) and in the amount of nicotinic receptors (*11*) and transsynaptic, cholinergically stimulated dopamine activity (*12*) have also been reported.

These biochemical studies, when considered with the cholinergic model for memory deterioration in old age, logically suggest that geriatric cognition might be improved by providing abundant amounts of choline or lecithin. However, clinical studies have failed to demonstrate reliable or therapeutically relevant effects in the cognitively impaired elderly (*13*). In fact, there is no empirical evidence that, by altering precursor availability, we can induce in the aged brain any of the changes that reportedly have been induced in the young healthy brain; or that any consistent behavioral changes occur at all. Although it is still too early to determine whether methods utilizing precursor control of the cholinergic system will be developed to reverse age-related memory impairments, other heuristically interesting possibilities should also be examined. For example, if age-related changes in the cholinergic system are at least partially responsible for the memory impairments, and if dietary manipulation of choline significantly affects cholinergic function, then it might be possible to modulate the rate at which the memory impairments occur with age by varying the availability of dietary choline.

We tested C57B1/6j mice of various age groups for memory of a one-trial, passive avoidance task either 24 hours or 5 days after training (Fig. 1). The results (Fig. 2) demonstrate that aged mice suffer impairments in learning and memory that appear similar to those found in aged rats, monkeys, and humans (*1, 3, 6*). After this "life-span" test, two different groups of retired breeder mice (8.5 months old) were given free access to purified diets that were either choline-deficient or choline-enriched (*14*). Because the life-span tests revealed that reliable passive avoidance deficits are not present at this age, it seems reasonable to assume that the major neurochemical

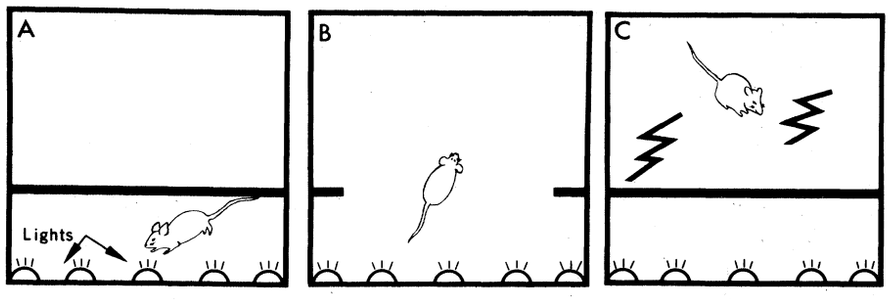

Fig. 1. Schematic representation of apparatus and procedure used to assess retention of passive avoidance across the life-spans of the mice and between choline groups. For training, each mouse was placed in the front chamber of a two-chambered apparatus (A). After a brief orientation period, a partition was raised, allowing the mouse to freely explore the apparatus, during which time it soon entered the second, darker chamber (B). Once the mouse was inside the second chamber, the partition was quickly lowered and a 0.3-mA shock was applied to the floor grids for 3 seconds (C). After this single training trial, the mouse was returned to its home cage to await testing for retention 24 hours or 5 days later. Testing was accomplished in exactly the same manner as the training, except the latency for entering the rear chamber was now measured. Pilot data demonstrated that if mice were not shocked on the training trial, latencies for entering the rear chamber were very low. However, if the mice were shocked on the training trial, latencies were consistently higher, but decreased with time. Thus higher latencies on the test day presumably reflect greater retention of the passive avoidance training trial.

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