MODULATION OF TUMOR DEVELOPMENT BY BUTYLATED HYDROXYTOLUENE IN EXPERIMENTAL ANIMALS

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INTRODUCTION

Carcinogenic Effects of BHT

The hindered phenolic antioxidant butylated hydroxytoluene, BHT (3,5-d-t-butyl-4-hydroxytoluene), is a widely used food additive. The general toxicology of BHT has been reviewed (Babich, 1982; Malkinson, 1983; Kahl, 1984, Ito *et al.*, 1985, Grice, 1986). BHT is considered to be a "generally recognized as safe" (GRAS) substance, not likely to have any untoward effects in man below the acceptable daily intake level of 0.05 mg/kg of body weight. Concerns about a possible carcinogenic effect of BHT have been raised on several occasions. A clear-cut carcinogenic effect for BHT has never been unequivocally demonstrated. In the mouse lung tumor assay, BHT did not elicit a positive response (Stoner *et al.*, 1973). In a bioassay of BHT for carcinogenicity in F344 rats and B6C3F$_1$ mice it was found that alveolar/bronchiolar carcinomas occurred at significant incidence in the lower dose group (mice exposed to 0.3% of BHT in the diet), but not in the higher dose group (NIH, 1979). In 2 later studies, no evidence was found for carcinogenicity of BHT at dietary doses ranging from 0.02% to 1.0% in Wistar rats or in B6C3F$_1$ mice (Hirose *et al.*, 1981; Shirai *et al.*, 1982). A chronic feeding study with BHT in rats appeared to suggest hepatocarcinogenic potential for BHT. The interpretation of the study was confounded by a significantly increased lifespan in the BHT fed animals, since at the time of appearance of the liver tumors most control animals already had died (Wurtzen and Olson, 1986). BHT in the diet did enhance the development of tumors in mouse strains having a particularly high background in spontaneously occurring tumors: liver tumors in C3H mice (Lindenschmidt *et al.*, 1986) and in B6C3F$_1$ mice (Inai *et al.*, 1988) and lung tumors in A/J mice (Witschi, 1985) or inbred Swiss mice (Maru and Bhide, 1982). In mouse skin, BHT is devoid of tumor initiating activity (Sato *et al.*, 1987). Altogether, results of the different studies do not tend to implicate BHT as a carcinogenic agent. However, they do leave some unresolved doubts about its safety (Blumenthal, 1986).

Tumor Modulating Effects of BHT

While data on a possible carcinogenic effect of BHT remain conflicting, there is ample experimental evidence to show that the antioxidant is capable of modifying the development of chemically induced tumors. Initially, the bulk of the evidence suggested that BHT might actually prevent tumor development.

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Together with other antioxidants, BHT was considered to be an effective chemopreventive agent for cancer (Wattenberg, 1980). However, in 1977 it was shown, independently in 2 laboratories, that BHT also could have the opposite effect. BHT enhanced both the development of liver tumors in rats (Peraino *et al.*, 1977) and of lung tumors in mice (Witschi *et al.*, 1977), provided exposure to the antioxidant occurred after exposure to the carcinogen. Since then, the "promoting" activity of BHT has been and continues to be examined in numerous experiments.

The enhancement of mouse lung tumor development by BHT has been most intensively studied. However, for the purpose of the present discussion, it seems to represent a special case. In most, although not all experiments, BHT was given to the mice by intraperitoneal injection rather than being administered in the feed. This makes this model system not directly applicable to the human situation. A "promoting" effect of BHT in mouse lung has been observed only in mouse strains that tend to develop a high spontaneous incidence of lung tumors. Other strains are much more resistant. The outcome of any experiment may depend upon experimental design. Intraperitoneal injection of BHT before administration of a lung specific carcinogen inhibits tumor development, whereas exposure to BHT following the carcinogenic stimulus usually increases tumor yield (Malkinson, 1985). Finally the role of metabolism in lung tumor promotion is beginning to be understood (Thompson *et al.*, 1991). There is good evidence to show that the tumor promoting effects of BHT in mouse lung involve selective cytotoxicity and possibly altered signal transduction. The particular model can be used to advantage to study the importance of BHT metabolism and of cellular/molecular events underlying the development of lung tumors in mice.

Scope of the Present Paper

The available data on the tumor-modulating effects of BHT in a variety of tissues and organs of rats and mice are analyzed. Examples were found for both tumor enhancement by BHT and for an inhibitory action of the antioxidant on tumor formation. A total of 33 papers describing such an effect were examined. Selection was limited to papers where BHT was administered to experimental animals in the diet; all other routes of exposure were excluded. Accordingly, the bulk of available data on tumor promotion in mouse lung by BHT was ignored in the present analysis, although this particular system offers the most insight into possible mechanisms (Malkinson, 1989; Witschi *et al.*, 1989).

METHODS

The literature on tumor-modulating effects of BHT in a variety of experimental systems is extensive. An attempt was made to interpret the available data. In order to accomplish this, the following procedure was adopted.

1. Selection of Studies

As a study we define, for the purpose of the present analysis, a single paper

published in the open literature that describes the effects of dietary BHT on chemical carcinogenesis.

2. Selection of Experiments

Each paper was scanned for individual experiments. In order to qualify and to be tabulated as an experiment, the following information had to be available: incidence of tumors in a specific site in animals treated with a chemical carcinogen and fed BHT, compared to the incidence of tumors in the same location in a corresponding control group (given the same dose of carcinogen, but fed a control diet). Tumor incidence is defined as number (percentage) of tumor bearing animals per total number of animals at risk.

3. Selection of Endpoints

Data were tabulated only when the listed endpoint was a tumor (malignant or benign). Whenever possible, the tabulation given in the original paper was used. Experiments that describe and quantitate the development of hyperplastic foci in the liver or hyperplastic lesions in other organs (e.g. stomach, bladder) were not included. An exception to this rule was made for the pancreas, where the only available evidence on the effects of BHT deals with the development of preneoplastic foci.

4. Classification of Experimental Data According to Protocol

Experiments were classified into 2 groups: S = experiments in which feeding of BHT preceded exposure to the carcinogen or in which carcinogen administration occurred simultaneously with feeding of BHT (e.g., carcinogen either given in the diet, drinking water or being injected while the animals were fed BHT) and A = experiments in which feeding of BHT began only after carcinogen exposure had been terminated.

5. Statistics

Differences in tumor incidence between BHT fed animals and corresponding controls were calculated for all tabulated incidence data by simple Chi-square analysis (not corrected for continuity).

RESULTS

In the survey of a total of 33 studies, an over-all animal population of 4667 rats and of 1697 mice was identified. In fact, these numbers overestimate the number of animals that were actually used in the 33 examined studies. This is due to the particular that in many studies animals developed tumors in several different sites. Since the analysis was done by tabulating experiments (i.e., tumor incidence in a specific organ) and since in the original studies the same animals were often tabulated repeatedly to display results pertaining to tumors in different organs, the total number of animals that were actually used is lower.

TABLE 1. Overall analysis of data.

	Rats	Mice
a) Over-all		
No. of studies examined [1]	27	6
No. of experiments [2]	104	37
Total No. of animals in studies	4667 (100%)	1697 (100%)
No. of animals fed BHT	3082 (66%)	896 (53%)
b) BHT vs. controls		
No. of animals fed BHT [3]	3082 (100%)	896 (100%)
No. of animals fed BHT bearing tumors	1225 (39%)	407 (45%)
No. of animals fed control diet [4]	1585 (100%)	801 (100%)
No. of control animals bearing tumors	637 (40%)	400 (49%)
c) Experiments showing an effect of BHT		
No. of experiments conducted	104 (100%)	37 (100%)
No. of experiments showing no effect of BHT	61 (59%)	29 (78%)
No. of experiments showing an enhancing effect of BHT	16 (15%)	5 (14%)
No. of experiments showing an inhibitory effect of BHT	27 (26%)	3 (8%)

1) Studies being defined as a paper published in the open literature.

2) Experiment being defined by data on tumor incidence in a specific organ and with all animals receiving the same dose of carcinogen and being exposed to BHT or to a control diet.

3) No. of animals in all experiments and being fed BHT.

4) No. of animals in all experiments and being fed a control diet

In all studies, the animals had been treated with a carcinogenic dose of a chemical carcinogen and were therefore at risk to develop cancer in one or several organs. The effects of BHT were assessed by offering the antioxidant to the carcinogen-treated animals in the diet in concentrations ranging from 0.03% to 1.0%. In the rat studies, 66% of the carcinogen-treated animals were fed BHT. Of all the mice, 53% received BHT (Table 1). The slight imbalance in the number of BHT-treated animals vs. controls is explained by the fact that in several studies one group of animals only served as controls for several BHT-treated groups. The overall effect of BHT on tumor development in the total animal population was calculated and

tumor incidence was compared to the one found in animals exposed to carcinogen and fed a control diet. It was found that in BHT-treated rats 39.7% of all animals at risk developed tumors, whereas in the controls 40.2% were found to have tumors. In mice, the corresponding numbers were 45.4% (animals fed BHT) and 49.9% (control diet).

The apparent no-effect of BHT in the overall study population is not surprising when the impact of BHT within single experiments is analyzed. Within the 33 published studies, 104 experiments involving rats and 37 experiments involving mice were identified. In the majority of all individual experiments, the difference between controls and BHT-treated animals was statistically not significant. In 15% of the rat experiments, BHT significantly enhanced tumor development. In 26% of the experiments BHT had a protective effect. In mice, significant enhancement was seen in 14% of all experiments and protection was found in 8%.

The next step was to examine the effects of BHT in every single organ system. Data on tumor incidence in 13 different sites were collected: liver, mammary gland, adrenals, stomach, upper gastrointestinal tract, colon, bladder, kidney, thyroid, ear duct, esophagus, lung and pancreas. After initial tabulation and examination of the data, it was recognized that the most important variable was the sequence of exposure to the carcinogen and to BHT. Accordingly, the individual experiments were separated into 2 groups: experiments in which BHT was administered to the animals either before and/or simultaneously with carcinogen treatment (Group S) and experiments in which the carcinogen was administered first, followed by BHT only after carcinogen exposure had ceased (Group A). The data are listed in Tables 2-10.

Tumor Data in Individual Organs

Liver (Tables 2 and 9). BHT modulated tumor development in 11 out of 28 experiments (39%). In all but one experiment BHT protected against hepatocarcinogenesis when given to rats in the diet simultaneously with a carcinogen. On the other hand, the original observation made by Peraino *et al.*, (1977) was confirmed: BHT did enhance tumor development if fed after carcinogen exposure. In mice, BHT did not modulate liver tumor development.

Mammary gland (Table 3). Only data from rats were available. The 7 statistically significant experiments out of a total of 21 (33%) show that BHT had a protective effect. Sequence of exposure was of no consequence.

Stomach, gastrointestinal tract and colon (Tables 4, 5 and 9). Only few studies (8 out of 32 or 25%) yielded a statistically significant result. However, they confirmed in both rats and mice that feeding of BHT during carcinogen exposure protects, whereas treatment with a carcinogen first, followed by BHT, enhances tumor development.

TABLE 2. Modulation by BHT of chemically-induced liver tumor development in rats.

Species/ Strain	Carcinogen/ BHT level	Exposure	BHT	Controls	p	Ref.
Rat/F344	DBN/0.7%	S	16/16	8/20	<0.05**	9
Rat/F344	FAA/0.03%	S	56/59	56/56	NS	16
Rat/F344	FAA/0.1%	S	45/50	" "	<0.05*	16
Rat/F344	FAA/0.3%	S	37/51	" "	<0.05*	16
Rat/F344	FAA/0.6%	S	23/41	" "	<0.05*	16
Rat/SD	FAA/0.6%	S	24/32	" "	NS	16
Rat/SD	MeDAB/0.5%	S	3/27	38/42	<0.05*	4
Rat/CD	FAA/0.66%	S	4/20	7/10	<0.05*	27
Rat/CD	FAA/0.66%	S	0/20	0/10	NS	27
Rat/CD	OHFAA/0.66%	S	3/20	6/10	<0.05*	27
Rat/CD	OHFAA/0.66%	S	0/20	0/10	NS	27
Rat/F344	FAA/0.5%	S	2/15	9/10	<0.05*	27
Rat/F344	OHFAA/0.5%	S	11/22	7/11	NS	27
Rat/CD	DEN/0.66%	S	17/20	10/10	NS	27
Rat/CD	DEN/0.66%	S	19/20	10/10	NS	27
Rat/F344	FAA/0.6%	S	24/41	56/56	<0.05*	30
Rat/SD	FAA/0.5%	A	24/93	6/92	<0.05**	20
Rat/F344	FAA/0.03%	A	4/12	3/14	NS	15
Rat/F344	FAA/0.1%	A	3/12	" "	NS	15
Rat/F344	FAA/0.3%	A	6/12	" "	NS	15
Rat/F344	FAA/0.6%	A	8/12	" "	<0.05**	15
Rat/F344	DBN/1.0%	A	6/21	5/21	NS	5

TABLE 3. Modulation by BHT of chemically-induced mammary tumor development in rats.

Species/ Strain	Carcinogen/ BHT level	Exposure	BHT	Controls	p	Ref.
Rat/SD	DMBA/0.03%	S,A	45/60	22/30	NS	3
Rat/SD	DMBA/0.1%	S,A	39/51	" "	NS	3
Rat/SD	DMBA/0.3%	S,A	35/51	" "	NS	3
Rat/SD	DMBA/0.6%	S,A	17/39	" "	<0.05*	3
Rat/SD	DMBA/0.03%	S,A	16/60	12/30	NS	3
Rat/SD	DMBA/0.1%	S,A	20/60	" "	NS	3
Rat/SD	DMBA/0.3%	S,A	10/51	" "	<0.05*	3
Rat/SD	DMBA/0.6%	S,A	12/51	" "	NS	3
Rat/SD	DMBA/0.7%	S,A	13/30	15/30	NS	11
Rat/SD	DMBA/0.7%	S,A	9/30	23/30	<0.05*	11
Rat/SD	DMBA/0.7%	S,A	18/30	30/30	<0.05*	11
Rat/F344	FAA/0.66%	S	6/20	2/10	NS	27
Rat/F344	OHFAA/0.66%	S	7/20	7/10	NS	27
Rat/SD	NMU/0.3%	S	11/16	11/16	NS	12
Rat/SD	DMBA/0.3%	S	9/16	16/16	<0.05*	12
Rat/SD	DMBA/0.5%	S	13/25	22/25	<0.05*	18
Rat/SD	DMBA/0.25%	S	17/25	" "	NS	18
Rat/SD	DMBA/0.7%	A	20/25	21/24	NS	10
Rat/SD	DMBA/0.7%	A	1/25	10/25	<0.05*	10
Rat/SD	DMBA/0.5%	A	18/25	" "	NS	18
Rat/SD	DMBA/0.25%	A	17/25	" "	NS	18

TABLE 4. Modulation by BHT of chemically-induced tumor development in the stomach and gastrointestial tract in rats.

Species/ Strain	Carcinogen/ BHT level	Exposure	BHT	Controls	p	Ref.
a) Stomach tumors						
Rat/WI	MNNG/1.0%	S	7/19	9/11	<0.05*	25
Rat/F344	DBN/1.0%	A	0/21	0/21	NS	5
Rat/WI	MNNG/1.0%	A	0/20	0/18	NS	24
Rat/Wi	MNNG/1.0%	A	2/20	" "	NS	24
Rat/F344	DMH/1.0%	A	2/19	3/18	NS	22
Rat/F344	MNU/1.0%	A	1/17	0/22	NS	8
Rat/F344	DMH/0.5%	A	8/40	7/36	NS	14
b) Gastrointestinal tract tumors						
Rat/SD	DMH/0.66%	S	4/9	1/10	NS	1
Rat/SD	DMH/0.66%	S	5/9	8/10	NS	1
Rat/F344	AOM/0.66%	S	29/50	20/23	<0.05*	29
Rat/F344	AOM/0.66%	A	24/25	" "	NS	29
Rat/WI	MNNG/1.0%	A	3/20	1/18	NS	24
Rat/F344	DMH/0.5%	A	1/20	2/20	NS	14
Rat/F344	DMH/0.5%	A	12/20	8/19	NS	14
Rat/F344	DMH/0.5%	A	28/47	15/49	<0.05**	14
Rat/F344	DMH/0.1%	A	23/50	" "	NS	14

Bladder (Table 6). In about half of the experiments (6 out of 13) BHT had an impact in rats. BHT enhanced bladder tumor development, regardless of exposure sequence. No data on mouse bladder tumors were available.

TABLE 5. Modulation by BHT of chemically-induced colon tumor development in rats.

Species/ Strain	Carcinogen/ BHT level	Exposure	BHT	Controls	p	Ref.
Rat/SD	DMH/0.66%	S	9/9	10/10	NS	1
Rat/F344	DMH/0.5%	A	9/19	10/18	NS	23
Rat/F344	DMH/0.5%	A	10/20	4/20	<0.05**	14
Rat/F344	DMH/0.5%	A	14/20	11/19	NS	14
Rat/F344	DMH/0.5%	A	9/47	15/49	NS	14
Rat/F344	DMH/0.1%	A	25/50	" "	<0.05**	14
Rat/F344	DMH/0.5%	A	1/40	3/36	NS	14
Rat/F344	MNU/0.5%	A	1/27	1/29	NS	14
Rat/F344	MNU/0.1%	A	2/29	" "	NS	14

TABLE 6. Modulation by BHT of chemically-induced bladder tumor development in rats.

Species/ Strain	Carcinogen/ BHT level	Exposure	BHT	Controls	p	Ref.
Rat/F344	DBN/0.7%	S	0/16	2/20	NS	9
Rat/F344	FAA/0.6%	S	17/41	0/56	<0.05**	30
Rat/F344	FAA/0.03%	S	1/59	0/56	NS	16
Rat/F344	FAA/0.1%	S	1/50	" "	NS	16
Rat/F344	FAA/0.3%	S	9/51	" "	<0.05**	16
Rat/F344	FAA/0.6%	S	18/41	" "	<0.05**	16
Rat/F344	FAA/0.6%	S	19/32	" "	<0.05**	16
Rat/F344	BBN/0.25%	A	3/14	4/14	NS	6
Rat/F344	BBN/0.5%	A	2/14	" "	NS	6
Rat/F344	BBN/1.0%	A	8/13	" "	NS	6
Rat/F344	BBN/1.0%	A	13/24	5/26	<0.05**	7
Rat/F344	BBN/1.0%	A	1/22	2/24	NS	7
Rat/F344	BBN/1.0%	A	4/15	0/21	<0.05**	8

Kidney (Tables 7 and 9). In the few experiments reported, no effect of BHT was found.

Adrenals (Table 7). Data from only one study were available and BHT, given simultaneously with DMBA, protected against the development of adrenocortical nodules in rats.

Thyroid (Table 7). In two different studies with rats, BHT was found to enhance development of thyroid adenomas if given after carcinogen administration.

TABLE 7. Modulation by BHT of chemically-induced tumor development in kidneys, adrenals and thyroid in rats.

Species/ Strain	Carcinogen/ BHT level	Exposure	BHT	Controls	p	Ref.
a) Kidney						
Rats/F344	DHPN/1.0%	A	2/15	0/15	NS	19
b) Adrenals						
Rat/SD	DMBA/0.03%	S,A	41/56	19/28	NS	3
Rat/SD	DMBA/0.1%	S,A	35/51	" "	NS	3
Rat/SD	DMBA/0.3%	S,A	10/45	" "	<0.05*	3
Rat/SD	DMBA/0.6%	S,A	7/38	" "	<0.05*	3
Rat/SD	DMBA/0.03%	S,A	12/56	13/29	<0.05*	3
Rat/SD	DMBA/0.1%	S,A	10/54	" "	<0.05*	3
Rat/SD	DMBA/0.3%	S,A	4/49	" "	<0.05*	3
Rat/SD	DMBA/0.6%	S,A	6/47	" "	<0.05*	3
c) Thyroid						
Rat/F344	MNU/1.0%	A	11/17	7/22	<0.05**	8
Rat/F344	DHPN/1.0%	A	11/15	4/15	<0.05**	19

Ear duct (Table 8). In rats, BHT had a protective effect, regardless of sequence of exposure.

Esophagus (Table 8). In only one experiment (out of a total of four) did BHT enhance tumor development. The experimental protocol showing enhancement was administration of the carcinogen first, followed by BHT; the other three

experiments involving simultaneous exposure had no effect. All data pertain to rats.

TABLE 8. Modulation by BHT of chemically-induced tumors in the ear duct, esophagus, lung and of preneoplastic pancreatic foci in rats.

Species/ Strain	Carcinogen/ BHT level	Exposure	BHT	Controls	p	Ref.
a) Ear duct						
Rat/F344	AOM/0.66%	S	12/50	12/23	<0.05*	29
Rat/F344	AOM/0.66%	A	11/25	" "	NS	29
Rat/SD	DMBN/0.7%	A	2/25	10/24	<0.05*	10
b) Esophagus						
Rat/F344	DBN/0.7%	S	4/16	7/20	NS	9
Rat/CD	DEN/0.66%	S	10/20	4/10	NS	27
Rat/CD	DEN/0.66%	S	4/20	1/10	NS	27
Rat/F344	DBN/1.0%	A	16/21	0/21	<0.05**	5
c) Lung						
Rat/F344	DHPN/1.0%	A	1/15	2/15	NS	19
Rat/F344	MNU/1.0%	A	3/17	5/22	NS	8
d) Pancreas foci						
Rat/Wi	Azas/1.0%	S	decreased*			26
Rat/LEW	Azas/0.45%	S	decreased*			21
Rat/WI	Azas/1.0%	A	increased**			26

Lung (Tables 8 and 10). BHT had no effect on lung tumors in rats. In mice, simultaneous exposure reduced tumor incidence, whereas exposure after carcinogen injection enhanced tumor development. The observations confirm data obtained with intraperitoneal injections of BHT in the mouse lung tumor model (Malkinson, 1985). However, out of a total of 22 experiments, dietary BHT modulated tumor development only in 5 (23%). Administration of BHT by intraperitoneal injection is thus a more effective means to modulate lung tumor development in mice.

Pancreas (Table 8). Simultaneous exposure to azaserine and BHT reduced, while sequential exposure increased, the number of preneoplastic lesions in the pancreas of rats.

TABLE 9. Modulation by BHT of chemically-induced gastrointestinal tract and kidney tumors in mice.

Species/ Strain	Carcinogen/ BHT level	Exposure	BHT	Controls	p	Ref.
a) Stomach tumors						
Mouse/ICR	BaP/1.0%	S	10/20	13/19	NS	28
Mouse/A/H	BaP/0.5%	S	2/9	12/12	<0.05*	28
b) Gastrointestinal tract tumors						
Mouse/B	MNU/0.5%	A	10/22	6/24	NS	13
c) Colon tumors						
Mouse/B	DMH/0.75%	S	15/25	9/18	NS	2
Mouse/B	DMH/0.75%	S	12/35	15/20	<0.05*	2
Mouse/B	DMH/0.5%	A	9/28	3/30	<0.05**	13
Mouse/B	DMH/0.05%	A	0/43	" "	NS	13
d) Liver tumors						
Mouse/S	INH/0.5%	S	2/20	1/29	NS	17
Mouse/S	HS/0.5%	S	0/28	2/29	NS	17
Mouse/B	DMH/0.5%	A	2/28	4/30	NS	13
Mouse/B	DMH/0.05%	A	6/43	" "	NS	13
Mouse/B	DMH/0.5%	A	8/41	3/27	NS	13
Mouse/B	DMH/0.05%	A	7/46	" "	NS	13
e) Kidney tumors						
Mouse/B	DMH/0.75%	S	1/25	0/18	NS	2
Mouse/B	DMH/0.75%	S	7/35	2/20	NS	2

Evidence for Dose-response

An attempt was made to establish whether there was evidence for a dose-response to BHT in the different experiments. For this, the individual experiments were separated into 4 groups, representing 4 different dose levels of BHT given in the diet. Results were counted as being positive when the experiment clearly showed

a BHT effect, regardless of whether BHT increased or decreased tumor incidence. Table 11 shows the percentage of positive experiments within a given dose range. The length of BHT exposure was not taken into consideration. The percentages of positive results were very similar at dietary BHT concentrations ranging from 0.25% to 1.0%; only at 0.1% and lower was there a diminished response to BHT. The lowest dose of dietary BHT used was 0.03%. In 1 out of 6 experiments, BHT had an effect at this level (reduced formation of adrenocortical nodules in rats treated with DMBA).

TABLE 10. Modulation by BHT of chemically-induced lung tumor development in mice.

Species/ Strain	Carcinogen/ BHT level	Exposure	BHT	Controls	p	Ref.
Mouse/SWR	INH/0.5%	S	3/20	15/29	<0.05*	17
Mouse/SWR	HS/0.5%	S	19/28	22/29	NS	17
Mouse/B	DMH/0.75%	S	11/25	10/18	NS	2
Mouse/B	DMH/0.75%	S	10/35	9/20	NS	2
Mouse/A/J	MCA/0.5%	A	24/24	27/29	NS	33
Mouse/A/J	MCA/0.5%	A	17/20	12/28	<0.05**	33
Mouse/A/J	MCA/0.5%	A	13/24	8/29	<0.05**	33
Mouse/A/J	MCA/0.5%	A	10/26	4/30	<0.05**	33
Mouse/A/J	BaP/0.5%	A	30/30	29/29	NS	33
Mouse/A/J	BaP/0.5%	A	15/27	11/30	NS	33
Mouse/A/J	BaP/0.5%	A	8/28	8/30	NS	33
Mouse/A	U/0.75%	A	24/24	26/26	NS	31
Mouse/A	U/0.75%	A	10/11	16/17	NS	31
Mouse/A	U/0.75%	A	23/23	23/28	NS	31
Mouse/A	U/0.75%	A	25/25	25/25	NS	32
Mouse/A	U/0.75%	A	22/25	12/25	<0.05**	32
Mouse/A	U/0.75%	A	10/26	11/25	NS	32
Mouse/A	U/0.75%	A	6/25	7/25	NS	32
Mouse/A	U/0.75%	A	10/10	21/21	NS	32
Mouse/A	U/0.75%	A	12/15	17/22	NS	32
Mouse/A	U/0.75%	A	12/18	11/23	NS	32
Mouse/A	U/0.75%	A	10/19	8/24	NS	32

TABLE 11. Dose-response to BHT in the different experiments.

% BHT in diet	Rats			Mice		
	No.[1]	Positive[2]	%	No.[1]	Positive[2]	%
1%	19	8	42.0%	1	0	0%
0.6%-0.7%	37	18	48.5%	17	2	11.8%
0.5%	17	6	35.0%	16	6	37.5%
0.25%-0.3%	14	7	50.0%	0	0	
0.03-0.1%	17	4	23.5%	3	0	0%

[1] Number of experiments conducted at this particular range of concentrations of BHT in the diet fed to the animals. Duration of exposure not considered.

[2] Number of experiments showing a statistically significant difference between BHT fed animals and controls, regardless of whether BHt mitigated or enhanced tumor development.

Explanations of Tables 2 to 10

Tables 2 to 10 list the actual incidence of tumors in various tissues and organs of rats and mice treated with a carcinogen and fed BHT or kept on a control diet. The individual columns list the following:

Species/Strain: indicates animal species and strain used in any particular experiment. The following abreviations for animal strains are used:

A/H	A/HeJ mice
A/J	A Mice, Jackson Laboratories
B	Balb/c mice
CD	CDSPF Charles River rate
F344	Fisher344 rats
ICR	Ha/ICR mice
LEW	Charles River rats
SD	Sprague-Dawley rats
SWR	Swiss-Webster mice
WI	Wistar rats

Carcinogen/BHT Level: The first letters denote the chemical carcinogen used to initiate the animals; abbreviations are given below. The number denotes the concentration of BHT used in the diet fed to the animals.

AOM	Azoxymethane
Azas	Azaserine
BaP	Benzo(a)pyrene
BBN	N-butyl-N-(4-hyrdroxybutyl)nitrosamine
DBN	N,N-dibutylnitrosamine
DEN	N-diethylnitrosamine
DHPN	Dihydroxy-d-n-propyl nitrosamine
DMBA	7,12 dimethylbenz(a)anthracene
DMH	1,2-dimethylhydrazine
FAA	N-2-fluorenylacetamide
HS	Hydrazine
INH	Isoniazid
MCA	3-methylcholathrene
MeDAB	3'methyl 4-dimethylaminoazobenzene
MNNG	N-methyl-N'nitro-N-nitrosoguanidine
MNU	Methylnitrosourea
OHFA	N-hydroxy-N-2-fluorenylacetamide
U	Urethan

Exposure: S denotes that the animals were fed BHT either before and/or during exposure to the carcinogen. A means that animals were exposed to the carcinogen first and only were fed BHT after BHT exposure had ceased.

BHT and Controls: The numbers indicate the number of tumor bearing animals per total number of animals in any given experimental group.

p: NS means no statistically significant difference between animals exposed to BHT and controls. <0.05** indicates that BHT significantly increased tumor incidence in BHT fed animals compared to controls; <0.05* indicates that BHT significantly inhibited tumor development.

Ref: The numbers refer to the following references listed in the bibliography:

1. Barbolt and Abraham, 1979
2. Clapp *et al.*, 1979
3. Cohen *et al.*, 1982
4. Daoud *et al.*, 1980
5. Fukushima *et al.*, 1987a
6. Fukushima *et al.*, 1987b
7. Imaida *et al.*, 1983
8. Imaida *et al.*, 1984
9. Imaida *et al.*, 1988
10. Ito *et al.*, 1986

11. King *et al.*, 1979
12. King *et al.*, 1981
13. Lindenschmidt *et al.*, 1986
14. Lindenschmidt *et al.*, 1987
15. Maeura and Williams, 1984
16. Maeura *et al.*, 1984
17. Maru and Bhide, 1982
18. McCormick *et al.*, 1984
19. Moore *et al.*, 1986
20. Peraino *et al.*, 1977
21. Roebuck *et al.*, 1984
22. Shirai *et al.*, 1984
23. Shirai *et al.*, 1985
24. Takahashi *et al.*, 1986
25. Tatsuta *et al.*, 1983
26. Thornton *et al.*, 1989
27. Ulland *et al.*, 1973
28. Wattenberg, 1973
29. Weisburger *et al.*, 1977
30. Williams *et al.*, 1983
31. Witschi, 1981
32. Witschi and Morse, 1983
33. Witschi, 1985

Summary of Data

Table 12 presents a summary of all experiments. Experiments were classified into 2 groups: those in which BHT had been given simultaneously with the chemical carcinogen (S) and those where the carcinogen was given first, followed by BHT (A). The animals from all experiments describing tumor development in the individual organs were added up, regardless of whether the experiment had shown a statistically significant effect or not. Tumor incidence in the BHT-treated animals was compared to tumor incidence in the controls. In general, the cumulative data agree well with the conclusions drawn from analysis of tables 2 to 11. Simultaneous exposure to BHT and a carcinogen mitigated development of tumors in rat liver, rat mammary gland, rat stomach, rat pancreas, rat adrenals, mouse stomach and mouse lung. Feeding of BHT following carcinogen exposure enhanced tumor development in rat liver, rat colon, rat pancreas, rat thyroid, rat esophagus and mouse lung. In two organs, the rat urinary bladder and the rat ear duct, BHT increased tumor development regardless of sequence of administration.

DISCUSSION

This paper describes a simple analysis on the overall impact of dietary BHT in rats and mice treated with a carcinogen. Obviously, the methodology used is very crude and only a few simple yes-no answers were developed with Chi-square statistics. Data collected in tables 2-10 and further consultation of the original

TABLE 12. Summary of all data.

Organ	Group	BHT	%	Controls	%	Significant
Rat liver	S	284/474	59.9	207/255	81.0	Yes
	A	51/162	31.5	14/127	11.0	Yes
Rat mammary	S	297/635	46.8	160/227	70.5	Yes
	A	56/100	56.0	31/49	63.3	No
Rat stomach	S	7/19	36.8	9/11	81.8	Yes
	A	13/137	9.5	10/115	8.7	No
Rat GI tract	S	38/68	55.9	29/43	67.4	No
	A	91/182	50.0	26/106	24.5	Yes
Rat colon						
	S	9/9	100.0	10/10	100.0	No
	A	71/252	28.2	44/171	25.7	No
Rat bladder	S	65/290	22.4	2/132	1.5	Yes
	A	31/102	30.4	11/85	12.9	Yes
Rat kidney	A	2/15	13.3	0/15	0.0	No
Rat adrenals	S	125/396	31.6	32/57	56.1	Yes
Rat thyroid	A	22/32	68.7	11/37	29.7	Yes
Rat ear duct	S	12/50	24.0	12/23	52.1	Yes
	A	13/50	26.0	12/24	52.1	Yes
Rat esophagus	S	18/56	32.1	12/40	30.0	No
	A	16/21	76.2	0/21	0.0	Yes
Rat lung	A	4/32	13.5	7/37	18.9	No

TABLE 12. Summary of all data (cont'd.).

Rat pancreas	S	decreased (2 experiments)				
	A	increased (1 experiment)				
Mouse stomach	S	12/29	41.3	25/32	78.0	Yes
Mouse GI tract	A	10/22	45.5	6/24	25.0	No
Mouse colon	S	27/60	45.0	24/38	63.2	No
	A	9/71	12.7	3/30	11.1	No
Mouse liver	S	2/48	4.2	3/58	5.2	No
	A	23/158	14.6	7/57	12.3	No
Mouse lung	S	43/108	39.8	56/96	58.3	Yes
	A	281/400	70.3	276/466	59.2	Yes

In this table, data from all experiments are summarized, regardless of whether a particular experiment showed a statistically significant effect or not. The different colmns indicate:
Organ: Organ or tissue that was analyzed for tumors.
Group: S denotes experiments in which BHT was fed concomitantly with the carcinogen; A denotes experiments where carcinogen exposure occurred first, followed by BHT administration.
BHT: Tumor data from animals fed BHT: number of tumor bearing animals/total number of animals.
Controls: Tumor data from animals fed control diet: number of tumor bearing animals/total number of animals.
Percentage %: Percentage of tumor bearing animals per total number of animals at risk.
Significant: Yes if difference between BHT fed animals and control animals is statistically significant at the level of $p < 0.05$, regardless of whether BHT enhanced or mitigated tumor developent (Chi-square test, not corrected for continuity).

papers might provide material for a more sophisticated analysis. Multiple possible variables were ignored. They include, but may not be limited to such considerations as strain and sex of experimental species; interdependence of tumor development in the same animals in different organs; duration of BHT exposure and dosimetry related to actual BHT intake; chemical class of carcinogens; dose of carcinogens and group sizes used in the individual experiments. The two last points might be particularly important. In any experiment that attempts to study modulation of tumor development, it is desirable to deal with a tumor incidence that allows one to detect either an

inhibitory or an enhancing effect of the putative modulatory agent. The likelihood that such experiments will yield conclusive results is dictated by statistical power. Statistical power is not only a function of the number of animals used but also of the anticipated tumor incidence. It is possible that many of the negative experiments might have yielded more definite results through better selection of different carcinogen doses and more animals per group.

With these limitations in mind, three simple questions were addressed: would BHT have an overall effect in the general population of animals studied; what effect had BHT in various organs, particularly if the sequence of exposure to carcinogen and BHT was taken into account as an important variable; and was there evidence for a dose-response?

The simplest way to look at the data is to reduce all observations to the most common denominator. A sizeable number of rodents were treated with a carcinogen at doses intended to produce tumors in various organs. Slightly more than half of the animals were exposed to BHT in the diet. Overall tumor incidence in all animals fed BHT was 41.0%, whereas in the population not given BHT it was 43.5%. If we knew nothing more about a large population of animals than that they were exposed to a chemical carcinogen, then we might have to conclude that BHT has no discernable effect. In addition, it is obvious that a BHT effect, whether positive or negative, was only found in a minority of all individual experiments. We also would have to conclude that above a concentration of 0.1% of BHT in the diet there does not exist a dose-response relationship. Although the overall response to BHT is diminished at dietary levels of 0.1% or less, no evidence for a threshold level was found.

A more helpful conclusion can be reached if the study population is split into animals that received BHT either before or concomitantly with a chemical carcinogen and into animals where carcinogen exposure occurred first, to be followed by BHT only after carcinogen exposure had ceased. In all organs, except in the bladder, concomitant exposure protects against tumor development. The only exception to this rule is a single experiment on tumor modulation in rat liver (Imaida *et al.*, 1988) In rat bladder, sequence of exposure played no role. On the other hand, whenever BHT intake followed administration of a carcinogen, BHT enhanced tumor development. Two exceptions to this rule were also found: development of mammary tumors and of ear duct tumors in rats (Ito *et al.*, 1986).

We may conclude from this observation that the effects of BHT on tumor development are mediated by two different mechanisms. Protection is likely to be a result of BHT's well known property as an inducer of mixed function oxidases (Kahl, 1984). BHT appears to favor mechanisms of metabolic detoxification whenever the antioxidant and a chemical carcinogen are present at the same time. This explanation has been offered since the first studies were done on the effects of BHT on chemical carcinogenesis (Wattenberg, 1973; Ulland *et al.*, 1973). Analysis of the additional data gathered since then reinforces such a

conclusion. This hypothesis could be tested directly in experiments where the effects of simultaneous exposure to BHT and to a physical carcinogen such as radiation are examined.

Separation of carcinogen and BHT administration precludes direct interference with metabolism and a different mechanism is likely to exist. The original rationale for testing the hypothesis that BHT would enhance tumor development was the observation that BHT produces cell hyperplasia in certain organs (Peraino *et al.*, 1977; Witschi *et al.*, 1977). This remains a possible mechanism, although for lung it has been shown that BHT enhances tumor development even in the complete absence of cell proliferation (Witschi, 1986). It appears thus to be more likely that the tumor-enhancing effects of BHT are mediated by a BHT metabolite. A possible metabolite for tumor promotion in mouse lung has recently been identified (Bolton *et al.*, 1990; Bolton and Thompson, 1991). If excreted in substantial amounts in the urine, such a metabolite might be responsible for enhanced bladder carcinogenesis regardless of sequence of exposure. In all other tissues the effects of BHT on carcinogen detoxification might outweigh any promoting effect of its metabolites, provided BHT and carcinogen are present at the same time. If not, enhanced tumor development by metabolites becomes the driving force.

In view of these observations it seems to become important to have detailed information on BHT metabolism, particularly in man. Finally it should be recognized that the doses of BHT required to modulate tumor development are quite high. If fed a diet containing 0.5% BHT, rats consume on average 150-170 mg/kg of BHT per day (Lindenschmidt *et al.*, 1987). Mice on the same diet ingest approximately 400 mg/kg per day (Lindenschmidt *et al.*, 1986). If remains uncertain whether much lower intake, as encountered by man, produces any biological effects at all.

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