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Research Section

CARCINOGENICITY STUDY ON BUTYLATED HYDROXYTOLUENE (BHT) IN WISTAR RATS EXPOSED *IN UTERO*

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Abstract—Groups of 60, 40, 40 and 60 F_0 Wistar rats of each sex were fed a semi-synthetic diet containing butylated hydroxytoluene (BHT) in concentrations to provide intakes of 0, 25, 100 or 500 mg/kg body weight/day, respectively. The F_0 rats were mated and groups of 100, 80, 80 or 100 F_1 rats of each sex were formed from 40, 29, 30 and 44 litters, respectively. After weaning, the highest dose (500 mg BHT/kg/day) was lowered to 250 mg/kg/day for the F_1 rats. The numbers of litters of ten or more pups at birth decreased with increasing BHT dose. At weaning, treated F_1 rats had lower body weights than the controls, the extent of the reduction being dose related; the effect, which persisted throughout the study, was most pronounced in the males. The survival of BHT-treated F_1 rats of both sexes was significantly better than that of the controls. No significant changes attributable to BHT treatment were found in the haematological parameters. F_1 females on the highest dose showed an increase in serum cholesterol and phospholipids, and serum triglycerides were reduced in this group in both sexes. Dose-related increases in the numbers of hepatocellular adenomas and carcinomas were statistically significant (at $P < 0.05$ or lower) in male F_1 rats when all groups together were tested for heterogeneity or analysis for trend. The increase in hepatocellular adenomas and carcinomas in treated female F_1 rats was only statistically significant for adenomas (at $P < 0.05$) in the analysis for trend. All hepatocellular tumours were detected when the F_1 rats were more than 2 yr old. Tumours were found in many other organs of some of the treated rats, but their incidence was not significantly different from that in controls. The role of BHT in the development of hepatocellular tumours requires further elucidation.

INTRODUCTION

Butylated hydroxytoluene (BHT), widely used as an antioxidant in food and in several technical products, has been shown to induce reversible mixed-function oxidases and liver enlargement in rats (Crampton, Gray, Grasso & Parke, 1977) and peliosis, hepatocellular vacuolation, degeneration and necrosis in the livers of male mice (National Cancer Institute, 1979). It has also been found to increase the mitotic activity of liver cells in rats (Lane & Lieber, 1967). It influenced the synthesis of prothrombin (Takahashi & Hiraga, 1978a) and caused haemorrhagic death in rats fed diets containing 0.017 or 0.69% BHT, respectively (Takahashi & Hiraga, 1978b). At similar levels it has been found to induce nephropathy (Meyer, Blom & Olsen, 1978) and to interfere with the normal function of the thyroid (Søndergaard & Olsen, 1982). Mutagenic activity was not demonstrated in the Ames test (Joner, 1977), but malignant transformation was found in cultured fibroblasts exposed to BHT (Djurhuus & Lillehaug, 1982). BHT may enhance (Peraino, Fry, Staffeldt & Christopher, 1977; Witschi, Williamson & Lock, 1977) or inhibit (Ulland, Weisburger, Yamamoto & Weisburger, 1973; Wattenberg, 1972) tumour formation in rodents treated with carcinogenic substances, but so far it has not been reported to be carcinogenic *per se* in mice or rats (Hirose, Shibata, Hagiwara *et al.* 1981; National Cancer Institute, 1979; Shirai, Hagiwara, Kurata *et al.* 1982). These studies were carried out in one generation and were terminated within 2 years. The work reported here was a two-generation carcinogenicity study of BHT in rats, the F_1 generation being dosed for their entire lifespan.

EXPERIMENTAL

Animals and diet. Specified-pathogen-free 3-wk-old Wistar rats of both sexes were obtained from Møllegaards breeding Centre Ltd, Ll. Skensved. During the study animals were given diet and water *ad lib.* and were kept in stainless-steel wire cages (two males or females/cage), maintained at 23 ± 1°C and a relative humidity of 60 ± 5%, with air changes 6–8 times/hr and electric light from 21.00 to 09.00 hr. The BHT used (Toxolan "P", food-additive grade) was obtained from Cedisa, Spain. More than 99.5% of the compound was determined as BHT, and the specifications were in accord with those of the European Economic Community (1978) and Joint FAO/WHO Expert Committee on Food Additives (1980). The BHT was mixed into a semi-synthetic powdered diet (Meyer, Blom & Søndergaard, 1982) in concentrations adjusted according to food consumption. Diet was prepared every second week. The stability of BHT in the diet was examined four times during each of the feeding periods for the F_0 and F_1 generations. The actual levels of BHT in the prepared diets were a few percent less than the added amounts. Analyses for aflatoxins and volatile nitrosamines were negative at the levels of analytical sensitivity used (0.1 ppb).

Experimental design and conduct. Groups of 40, 40 and 60 F_0 rats of each sex were fed from 7 wk of age

Table 1. Reproduction data for F_0 rats fed diet containing BHT

Parameter		Data for groups of rats fed BHT in doses (mg/kg body weight/day) of: 0	25	100	500
No. of rats/group	—females	40	29	30	44
	—males	39	29	30	44
Gestation rate (%)		88	95	93	95
No. of pups/litter	—mean	10.9	9.6	10.3	9.1*
	—after standardization	8.0	8.0	8.0	7.9
	—at weaning	7.9	8.0	7.7	7.8
Body weight (g) of pups‡	—at birth	5.9	5.9	5.7†	5.7
	—at weaning	42.4	40.4†	39.7††	25.3††

‡Average of mean pup weight/litter.

Values marked with an asterisk or dagger(s) show a statistically significant difference from the control: *$P < 0.001$ by the Armitage–Cochran test for linear trend in proportions of litters with ten or more pups; †$P < 0.05$ and ††$P < 0.001$ by Student's t test.

to the end of the lactation period (females) on the semi-synthetic diet with BHT added at levels providing intakes of 25, 100 or 500 mg/body weight/day, respectively. A fourth group of 60 F_0 rats of each sex was given control diet. The F_0 rats were mated after 13 wk of dosing and groups of 100, 80, 80 or 100 F_1 rats of each sex were formed from 40, 29, 30 and 44 litters, respectively. After mating, the male F_0 rats were left out of the study; the females were omitted after weaning. Because of an adverse effect on the kidney (Meyer *et al.* 1978) in the female F_0 rats, the concentration of BHT given to the highest dose group was lowered to 250 mg/kg body weight/day for the F_1 generation.

Body weight was recorded weekly until the rats were 31 wk old and subsequently every second week. Food consumption was measured weekly. Blood samples from 20 F_1 males and females in the control and highest dose group were drawn from the orbital plexus under CO_2 anaesthesia after 9, 19, 43 and 108 wk. Haematocrit and haemoglobin were determined in whole blood, and red and white blood cell and differential white cell counts were made. Glucose, blood urea nitrogen, free and total cholesterol, triglycerides and phospholipids were measured in serum. All F_1 rats were inspected regularly for the presence of tumours. The study was terminated by killing the surviving rats at 141–144 wk of age. Gross and microscopic pathology was performed on these animals as well as on those that were killed or died during the entire study. Specimens from the liver, kidneys, heart, lungs, brain, spleen, pituitary gland, thyroid, thymus (if any), pancreas, adrenals, testes, ovaries, seminal gland, uterus, mesenteric and axillary lymph nodes, salivary gland, gastro-intestinal tract (six levels), urinary bladder, spinal cord, peripheral nerve, skeletal muscle, bone, skin, mammary gland, eye and Harderian gland were fixed in 10% neutral buffered formalin and embedded in paraffin, and sections were stained with haematoxylin and eosin for histological examination. Other appropriate staining methods were used for selected specimens. Animals that survived beyond wk 43, the time when the first tumour appeared in the spleen of a male rat in the high-dose group, were included in the 'effective numbers'.

Statistics. Student's t test was used for biochemical, haematological and other biological data for F_1 rats. The Armitage–Cochran test for linear trend was used for litterwise analysis of pre-weaning mortality. Data on mortality and tumour incidence in different groups were analysed according to Peto, Pike, Day *et al.* (1980). A test for intra-litter correlation was performed according to Grice, Munro & Krewski (1981).

RESULTS

F_0 rats: food consumption, body weights and reproduction data

No differences in food consumption were noted between BHT-treated and control rats. Male and female rats dosed with 500 mg BHT/kg/day showed a statistically significant ($P < 0.001$) reduction in body-weight gain, compared with the controls, from wk 6 of treatment, and this persisted throughout their life. Data on reproduction are shown in Table 1. The mating period was terminated within 1 wk and the gestation rate was comparable among the groups. The Armitage–Cochran test for linear trend in proportions demonstrated that the fraction of litters with ten or more pups decreased significantly with BHT dose ($P < 0.001$). No significant reduction in viability attributable to BHT was observed during the lactation period. The average birth weights of the F_1 pups in the middle- and high-dose groups were slightly lower than the average weight in the control group. During the lactation period the pups in the BHT-treated groups showed a dose-related depression of body-weight gain. Thus the body weight of pups at weaning was 5, 7 and 41% lower than that of the controls in the groups given 25, 100 and 500 mg BHT/kg/day, respectively.

F_1 rats

Food consumption and body weights. BHT administration had no effect on the clinical appearance or behaviour of the animals, apart from a slight reddish discoloration of the urine in males in the high-dose group. Data on food consumption, body weights and BHT intake are given in Table 2 and Fig. 1. No reduction in average food consumption was seen in any group given BHT. The dose-related depression of the mean body weight in the test groups compared with the controls at the end of the lactation period persisted throughout the study in both sexes. The lower body weights in F_1 rats given 250 mg BHT/kg differed from the control values by up to 21% for

Table 2. Mean body weight, food consumption and BHT intake up to wk 138 for F_1 rats fed a diet containing BHT

Nominal BHT intake (mg/kg body weight/ day)	Effective no. of rats	Mean body weight (g) at wk: 5	7	9	15	34	90	138	Mean food intake (g/rat/day)	Mean BHT intake (mg/kg body weight/ day)
Males										
0	100	105	186	243	357	471	575	487	23	0
25	80	103	181*	244	350	453**	550**	450*	22	25
100	80	97***	181*	243**	346*	447***	516***	433**	23	108
250	99	83***	150***	216***	301***	385***	459***	413***	22	276
Females										
0	100	86	140	176	231	277	344	313	18	0
25	79	86	139	176	227	268**	343	312	17	26
100	80	89*	139	176	226*	260***	319***	305	18	106
250	99	68***	122***	159***	208***	247***	288***	281**	18	287

Body weights marked with asterisks differ significantly (by Student's t test) from the corresponding control value: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

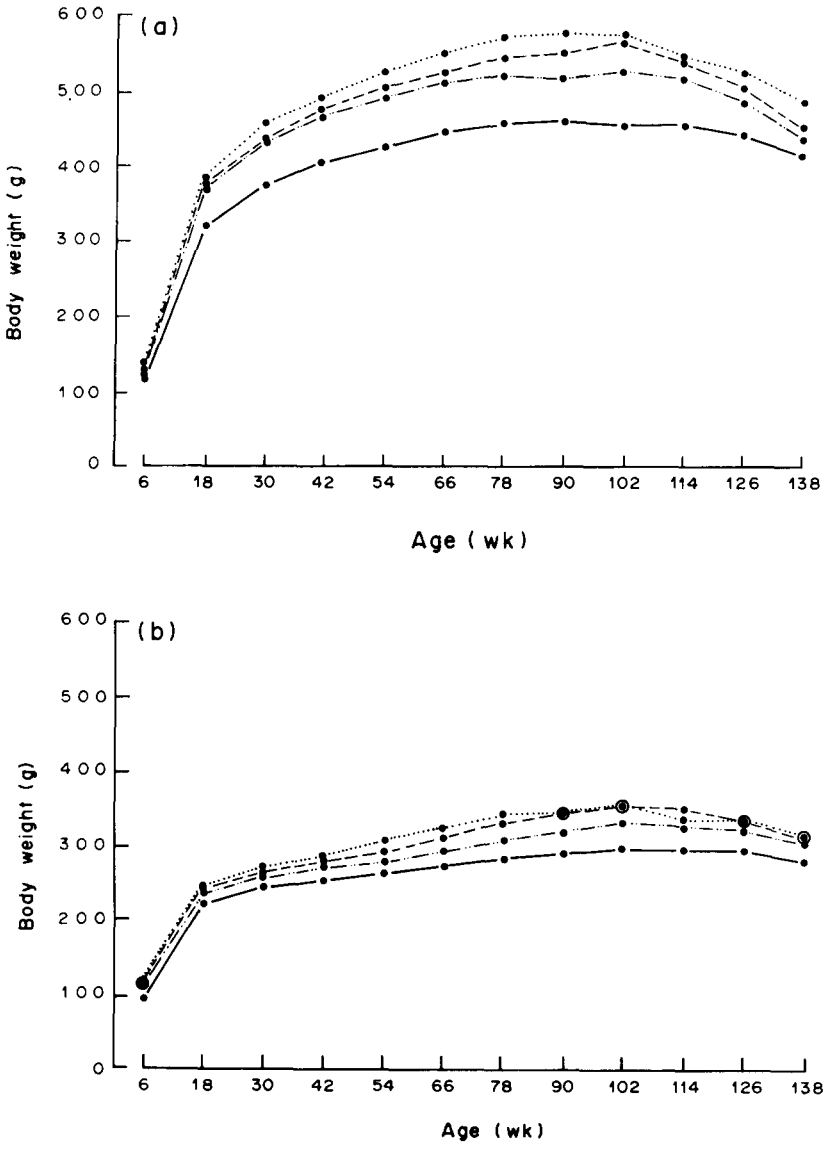

Fig. 1. Mean body weights of (a) male and (b) female F_1 rats given control diet (·····) or diet providing an intake of 25 (– – –), 100 (— · · —) or 250 (——) mg BHT/kg body weight/day.

Table 3. Mortality of F_1 rats fed a diet containing BHT

Group (mg BHT/kg/day)	Effective no. of rats	No. of rats dying during wk*: 0–90	91–104	105–113	114–118	119–126	127–132	133–140	Survivors killed at wk 141–144 No.	%
Males										
0	100	20	10	13	8	11	10	12	16	16
25	80	8	11	6	3	13	11	8	20	25
100	80	8	12	3	2	10	7	11	27	34
250	99	7	7	6	4	8	13	10	44	44
Females										
0	100	16	15	18	8	11	7	8	17	17
25	79	10	9	4	6	13	10	8	19	24
100	80	5	17	5	5	7	9	11	21	26
250	99	9	5	11	12	8	5	10	39	39

*Of age.

males and 16% for females. In the 100-mg/kg group these differences were up to 11% (males) and 10% (females) and in the 25-mg/kg group up to 7% (males) and 5% (females).

Survival. F_1 rats given BHT obviously survived longer than the controls (Table 3). By wk 104, 86% of males and females in the high-dose group had survived compared with 70% of the males and 69% of the females in the control group, and 44% of males and 39% of females in the high-dose group survived to termination (at wk 141–144 of age) compared with 16% of control males and 17% of control females. In both sexes significant differences ($P < 0.001$) in longevity were seen. The higher mortality up to 2 yr of age among control rats compared with those in treated rats mainly originated in males from inflammation of the bladder, often associated with stones, and in females from earlier occurrence of nephropathy and pituitary tumours.

Blood analyses. The haematological findings showed no persistent changes that could be attributed to BHT (data not tabulated). Table 4 shows that blood levels of cholesterol and phospholipids were higher in female rats treated with the highest level of BHT than in the controls, at least in the first year. Both sexes showed lower levels of triglycerides in the treated than in control rats at wk 19, 43 and 108. These results are, in principle, in accord with other studies (Hirose *et al.* 1981).

Pathology. The incidences of hepatocellular adenomas and carcinomas in males and of hepatocellular adenomas in females were higher in F_1 rats treated with BHT than in the control, as previously reported (Olsen, Bille & Meyer, 1983). All hepatocellular tumours were found incidentally. The first carcinoma in the treated rats was observed at wk 132 in a male in the highest dose group. The rest of the carcinomas were observed when the study was terminated. The only carcinoma in the controls was found in a male rat at 117 wk of age. The first adenoma was observed in a male in the high-dose group after 115 wk, but most adenomas in both sexes were found at termination (wk 141–144). No interim kill was performed. The incidences of hepatocellular nodular hyperplasia, adenomas and carcinomas and the related statistics are shown in Table 5. No intra-litter correlation was found for rats having a hepatocellular tumour. The times to the detection of hepatocellular neoplasia are given in Table 6. Grossly, the hepatocellular adenomas varied from 4 to 30 mm in diameter and the carcinomas were usually 15 mm or more in diameter. No preferential location of the neoplasms was observed in the liver. Ascites was occasionally seen in connection with large carcinomas. Histologically,

Table 4. Serum chemistry of F_1 rats fed a diet containing BHT

Serum constituent	Group (mg/BHT/kg/day)	Concn (nmol/litre serum) at wk (of age): 9	19	43	108
Males					
Free cholesterol	0	0.53 ± 0.02	0.64 ± 0.04	0.72 ± 0.03	1.13 ± 0.10
	250	0.64 ± 0.04*	0.68 ± 0.03	0.66 ± 0.03	1.10 ± 0.08
Total cholesterol	0	2.05 ± 0.09	2.31 ± 0.16	2.84 ± 0.13	4.14 ± 0.38
	250	2.27 ± 0.11	2.26 ± 0.10	2.45 ± 0.08*	3.82 ± 0.25
Phospholipids	0	2.82 ± 0.20	2.45 ± 0.07	2.93 ± 0.13	2.95 ± 0.21
	250	2.52 ± 0.02	2.35 ± 0.08	2.51 ± 0.09*	2.83 ± 0.20
Triglycerides	0	ND	1.67 ± 0.20	1.85 ± 0.22	1.76 ± 0.21
	250	ND	0.75 ± 0.08***	0.97 ± 0.12**	1.24 ± 0.17
Females					
Free cholesterol	0	0.58 ± 0.02	0.68 ± 0.03	0.75 ± 0.03	0.93 ± 0.06
	250	0.82 ± 0.04***	0.83 ± 0.04**	0.92 ± 0.05**	0.81 ± 0.04
Total cholesterol	0	2.02 ± 0.10	2.12 ± 0.10	2.72 ± 0.13	3.21 ± 0.19
	250	2.63 ± 0.11***	2.76 ± 0.11***	2.97 ± 0.15	2.81 ± 0.15
Phospholipids	0	2.74 ± 0.12	2.53 ± 0.08	3.21 ± 0.11	3.07 ± 0.22
	250	2.89 ± 0.07	2.99 ± 0.09***	3.33 ± 0.12	250 ± 0.14*
Triglycerides	0	ND	1.28 ± 0.15	2.02 ± 0.17	3.42 ± 0.38
	250	ND	0.97 ± 0.08	1.10 ± 0.12***	1.20 ± 0.10***

ND = Not determined

Values are means ± SEM for groups of 20 rats, except for the female control phospholipid level at wk 108 (19 rats) and those marked with asterisks differ significantly (by Student's t test) from the corresponding control: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Table 5. Occurrence of nodular hyperplasia, hepatocellular adenomas and carcinomas in Wistar rats fed a diet containing BHT

Group (mg/BHT/kg/day)	Effective no. of rats	No. of rats with: Nodular hyperplasia	Adenoma	Carcinoma
		Males		
0	100	2	1	1
25	80	0	1	0
100	80	2	5	1
250	99	2	18*	8†
		Females		
0	100	2	2	0
25	79	0	3	0
100	80	4	6	0
250	99	5	12‡	2§

*Overall test for heterogeneity, $P < 0.001$, chi-square = 18.17, 3 df. Test for trend, $P < 0.001$, chi-square = 17.97, 1 df.

†Overall test for heterogeneity, $P < 0.05$, chi-square = 11.12, 3 df. Test for trend, $P < 0.01$, chi-square = 9.40, 1 df.

‡Overall test for heterogeneity, not significant, chi-square = 5.20, 3 df. Test for trend, $P < 0.05$, chi-square = 4.99, 1 df.

§Overall test for heterogeneity, not significant, chi-square = 2.87, 3 df. Test for trend, not significant, chi-square = 2.59, 1 df.

hepatocellular lesions were classified according to the criteria described by the Institute of Laboratory Animal Resources (1980). The lesions identified by the terms nodular hyperplasia and hepatocellular adenoma used in this study are identical to those described for hyperplastic foci and areas and for hepatic cell adenoma, respectively. Basophilic adenomas were seen occasionally, but eosinophilic adenomas predominated. The hepatocellular carcinomas consisted of basophilic hepatocytes forming a trabecular pattern. In some carcinomas, projection of irregular cords without endothelial lining was seen in dilated sinusoids. However, no metastases of any carcinomas were detected grossly or microscopically. A careful search of transverse sections at five different levels of the lungs revealed no histological metastases either in the capillaries or in the pulmonary tissue. A high incidence of hepatocellular adenomas was seen in both sexes of rats in the highest dose group, but a higher number of hepatocellular carcinomas in the males than in the females in this group indicated a greater susceptibility in the males.

The numbers of tumours found in various organs of rats treated with BHT and in the controls are summarized in Table 7. A slight over-representation among treated rats was found for the following tumours: C-cell adenoma (females), C-cell carcinoma (males), islet-cell adenoma (males and females), exocrine adenoma of the pancreas (males), theca granulosa-cell adenoma, adenoma and adenocarcinoma of the uterus, thymoma (females), haemangioma and reticulum-cell sarcoma of the reticuloendothelial system (males) and ductular adenoma of the mammary gland (females). However, no statistically significant increases in incidence were recorded in the treated group compared with the control. The low incidence of theca granulosa-cell adenomas, limited to the group on the highest dose, was significant ($P < 0.05$) in analysis for trend. No corresponding increase in malignant tumours of the ovary was seen and the finding was judged to be incidental, without relation to BHT treatment.

The overall numbers of tumour-bearing animals, the numbers of rats with a malignant or a benign tumour, and the animals with one, two or multiple tumours are presented in Table 8. The overall numbers of F_1 rats bearing a malignant tumour (males and females) or multiple tumours (females) appears

Table 6. Time to detection of hepatocellular adenomas and carcinomas in F_1 rats fed a diet containing BHT

Group (mg BHT/kg/day)	Age (wk) at which tumour was detected: Adenomas	Carcinomas
	Males	
0	133	117
25	119	
100	136, 139, 141, 143, 143	141
250	115, 119, 125, 127, 138 141, 141, 141, 141, 141, 141, 142, 142, 142, 142 142, 143, 144	132, 141, 142, 142, 143 143, 143, 143
	Females	
0	117, 117	
25	132, 134, 143	
100	125, 129, 136, 142, 142, 143	
250	134, 135, 140, 140, 141 141, 142, 142, 142, 142 143, 143	141, 143

Table 7. Incidence of tumours (excluding hepatocellular tumours) in rats fed a diet containing BHT

Tumour site and type	BHT intake (mg/kg/day)...	No. of rats affected							
		Males				Females			
		0	25	100	250	0	25	100	250
	Effective no. of rats...	100	80	80	99	100	79	80	99
Brain									
Pinealoma		2	—	—	—	—	—	—	—
Astrocytoma		1	2	3	2	1	1	1	1
Ependymoma		1	—	1	—	—	—	—	—
Glioblastoma		—	—	1	1	1	—	—	—
Oligodendroglioma		—	—	—	1	—	—	—	—
Haemangioma		—	—	—	—	1	—	—	—
Unclassified glioma		—	—	—	1	—	1	—	—
Sarcoma		3	—	1	—	—	1	—	—
Pituitary gland									
Adenoma		39	31	26	29	58	47	53	56
Ganglioma		—	—	—	—	—	—	1	—
Carcinoma		2[b]	2	2	1	—	1	1	2
Thyroid gland									
C-cell adenoma		12	9	7	9	6	4	4	11
Papillary adenoma		—	1	—	2	2	—	—	1
Follicular adenoma		1	—	—	1	—	—	1	—
C-cell carcinoma		3	—	1	6	4	3	—	3
Follicular carcinoma		—	1	—	2	—	—	1	1
Parathyroid gland									
Adenoma		1	—	—	1	—	—	—	—
Carcinoma		—	1	—	—	—	—	1[d]	—
Adrenal gland									
Cortical adenoma		—	—	—	1	—	—	3	—
Medullary adenoma		4	1	1	2	1	—	—	1
Cortical carcinoma		—	—	—	—	1	—	—	—
Medullary carcinoma		8[h]	5	2	5	3	2	—	1
Malignant ganglioneuroma		—	—	—	—	1	—	—	—
Pancreas									
Islet-cell adenoma		9	6	11	17	3	6	2	6
Exocrine adenoma		8	8	10	12	4	—	4	5
Islet-cell carcinoma		1	1	—	—	—	—	—	—
Exocrine carcinoma		1	—	—	—	—	—	—	1
Testis									
Interstitial-cell adenoma		6	6	4	6				
Haemangioendothelioma		1	—	—	—				
Sertoli-cell adenoma		—	—	—	1				
Sertoli-cell carcinoma		—	—	—	1				
Seminal gland									
Adenoma		1	—	—	1				
Carcinoma		—	—	1	1				
Sarcoma		1	—	—	—				
Ovary									
Theca granulosa-cell adenoma						—	—	—	4
Thecoma						1	—	—	1
Granulosa-cell adenoma						—	—	1	—
Haemangiopericytoma						1	—	—	—
Theca granulosa-cell carcinoma						—	1	—	1
Granulosa-cell carcinoma						—	1	1	—
Uterus/vagina									
Fibromatous polyp						3	2	1	3
Adenoma						2	1	3	5
Leiomyoma						5	2	4	4
Fibroma						2	2	1	1
Squamous-cell carcinoma						—	—	1	—
Unclassified carcinoma						—	—	1	—
Leiomyosarcoma						—	—	—	1
Fibrosarcoma						—	1	—	—
Adenocarcinoma						3	4[h*]	5[fc]	7[h*]
Haemangioma						1	—	—	1
Urinary bladder									
Papilloma		12	1	—	2	1	—	1	—
Papillary carcinoma		3	—	—	—	—	—	—	—
Squamous-cell carcinoma		—	—	—	1	—	—	1	—
Squamous papilloma		—	1	—	—	—	—	—	—
Papillary adenoma		1	—	—	—	—	—	—	—
Kidney									
Hamartoma		—	—	—	—	—	1	—	—
Haemangioma		—	—	—	—	—	1	—	—
Unclassified sarcoma		—	—	—	1	—	—	—	—
Unclassified carcinoma		1	—	—	—	—	—	—	—

[*contd*]

Table 7—*continued*

Tumour site and type / BHT intake (mg/kg/day) / Effective no. of rats	No. of rats affected							
	Males				Females			
	0	25	100	250	0	25	100	250
	100	80	80	99	100	79	80	99
Oral cavity/oesophagus								
Papilloma	1	1	—	—	1	1	—	—
Squamous-cell carcinoma	—	1	—	—	—	—	—	1
Unclassified carcinoma	—	1[b]	—	—	—	—	—	—
Stomach								
Papilloma	—	1	—	—	—	—	—	—
Squamous-cell carcinoma	—	—	—	1	—	1	—	—
Intestine								
Adenoma	—	—	1	1	—	—	1	—
Lipoma	2	—	—	—	—	—	—	—
Leiomyoma	—	—	—	—	—	—	2	—
Leiomyosarcoma	2	—	1	—	1	2	1	1
Adenocarcinoma	—	3	2	1	—	1	—	—
Unclassified sarcoma	—	—	—	—	—	—	1	1
Unclassified carcinoma	—	—	2[d]	—	—	—	—	—
Liver								
Cholangioma	—	—	—	1	1	1	—	1
Haemangioendotheliosarcoma	—	—	—	1	—	—	—	—
Lung								
Bronchogenic carcinoma	1	3	—	1	1	2	1	—
Unclassified sarcoma	—	—	—	2[e]	—	—	—	—
Thoracic cavity								
Haemangioendotheliosarcoma	—	—	1	—	—	—	—	—
Liposarcoma	—	—	—	—	1[d]	—	—	—
Fibrosarcoma	—	—	—	—	—	—	—	1
Carcinosarcoma	—	—	—	—	—	—	—	1
Abdominal cavity								
Haemangioma	1	—	—	—	—	—	—	—
Lipoma	1	2	1	1	3	—	—	1
Mesothelioma	1	0	1	1	—	—	—	1
Fibroma	—	1	—	—	—	1	—	—
Haemangiopericytoma	—	—	—	—	1[g]	—	—	—
Malignant mesothelioma	—	—	—	—	1	—	—	—
Fibrosarcoma	—	2	1[h]	—	—	—	—	1[c]
Unclassified carcinoma	1	1	—	—	—	—	1	—
Skeleton								
Osteoma	—	—	—	—	—	—	—	1
Osteosarcoma	—	—	1	2	—	—	—	—
Unclassified sarcoma	—	—	—	1[b]	—	—	—	—
Lymphoreticular system								
Thymoma	—	1	1	—	1	2	2	4
Malignant thymoma	—	1	—	—	—	1[h]	1[d]	—
Plasmocytoma	—	—	—	—	1	—	—	—
Haemangioma	—	1	1	3	2	1	1	2
Fibrosarcoma	2	—	—	—	1	—	—	1
Reticulum-cell sarcoma	2	—	1	5[c]	—	2[h]	2[dc]	—
Lymphosarcoma	1[a]	—	1	1	1	—	—	2[h]*
Subcutaneous tissue								
Fibroma	4	4	—	—	1	3	1	—
Histiocytoma	—	—	1	—	—	—	—	—
Lipoma	1	—	1	1	—	1	—	—
Haemangioma	—	—	—	—	—	1	—	—
Haemangiopericytoma	—	—	—	1	—	—	—	—
Liposarcoma	1	—	—	—	—	—	—	—
Fibrosarcoma	2[b]	1	4[h]	2[d]	2	1	—	1
Reticulum-cell sarcoma	—	—	1[c]	—	—	—	—	—
Unclassified sarcoma	—	1[d]	1	1	—	—	—	—
Skin								
Papilloma	1	2	1	—	—	—	—	1
Basalioma	—	1	—	—	1	1	—	1
Keratoacanthoma	—	—	—	—	1	—	—	—
Squamous-cell carcinoma	5	1	2	2	—	3	—	2[d]
Basocellular carcinoma	—	—	—	1	—	—	—	—
Trichoepitheliocarcinoma	—	—	—	1	—	—	—	—
Adenocarcinoma	—	—	—	—	1	—	—	—
Muscle								
Haemangiosarcoma	1	1	—	—	—	—	—	—
Unclassified sarcoma	—	—	1	—	—	—	—	—
Mammary gland								
Ductular adenoma	—	—	—	—	—	1	—	3
Adenofibroma	1	3	1	1	12	9	11	7
Fibroadenoma	—	—	—	—	22	16	12	11

[*contd*]

Table 7—*continued*

Tumour site and type	BHT intake (mg/kg/day)...	No. of rats affected							
		Males				Females			
		0	25	100	250	0	25	100	250
	Effective no. of rats...	100	80	80	99	100	79	80	99
Carcinoma		—	1	—	—	—	2	1	2
Ductular adenocarcinoma		—	—	1	—	—	—	—	—
Fibrosarcoma		—	—	—	—	1	—	—	—
Salivary gland									
Carcinoma		—	—	—	—	—	—	—	1
Heart									
Osteosarcoma		—	—	1[h]	—	—	—	—	—
Fibrosarcoma		—	—	1	—	—	—	—	—
Reticulum-cell sarcoma		—	—	—	—	—	1	—	—
Eye									
Neuroganglioma		—	—	1	—	—	—	—	—
Sarcoma		—	—	—	1	—	—	—	—
Nervous tissue									
Neurofibrosarcoma		—	—	—	—	—	—	—	1

*Metastases were found in two of the rats.

Superscripts a–h indicate the finding of a metastasis to the (a) heart, (b) lung, (c) subcutis, (d) lymph nodes, (e) liver, (f) thymus, (g) intestine or (h) multiple organs, only one of the rats being so affected except where otherwise indicated (*).

to be slightly but not significantly enhanced in the high-dose group compared to corresponding numbers in the controls.

Among the non-neoplastic lesions in the liver, a dose-related increase in the incidence of bile-duct proliferation and cysts was found in males and of focal cellular enlargement in females (Table 9). Nephropathy and fibrosis of the heart were less frequent in BHT-treated rats than in the controls. The other non-neoplastic lesions occurred incidentally and showed no relationship to BHT treatment (Table 9).

DISCUSSION

BHT induced benign and malignant hepatocellular neoplasms in Wistar rats of both sexes under the conditions of this study. A dose-response relation was noted and was most pronounced in the males. In the high-dose group, the overall numbers of F_1 rats with a malignant tumour (males and females) or with multiple tumours (females) were slightly higher, but not to a statistically significant degree, than in the controls. In a study of Wistar rats by Hirose *et al.* (1981) the overall incidence of tumours was also slightly higher in BHT-treated groups.

Recently, the rationale of using *in utero* exposure in cancer bioassays has been reviewed (Grice *et al.* 1981). It is, however, unknown to what extent the sensitivity of the test is improved by following such a procedure. Use of the *in utero* exposure in our study extended the period of BHT exposure, since it has been shown that BHT crosses the placental barrier and is excreted in rat milk (J. Chr. Larsen, personal communication, 1983). The latter is in agreement with the findings of studies using cross-fostering; these demonstrated that untreated pups suckled by dams treated with 500 mg BHT/kg body weight/day gained less weight than pups suckled by untreated mothers (Meyer & Hansen, 1980).

The lower body weight of rats treated with BHT is important and it is questionable whether the lower body weight also found by others (Frawley, Kohn, Kay & Calandra, 1965; Hirose *et al.* 1981; National Cancer Institute, 1979) should be regarded as a 'toxic' effect. One might consider the lower body weight as an adaptive response. This suggestion is supported by the almost constantly lower and parallel body-weight curves of the treated rats throughout the entire study as well as by the comparable food conversion among the groups. Whether hyperactivity of the thyroid and consequently an increase in basal metabolism has any influence on the body weight of these rats is unknown. However, the iodine uptake and the relative weight of the thyroid gland were significantly increased in Wistar rats fed 5000 ppm BHT in the diet for 90 days (Søndergaard & Olsen, 1982).

The spontaneous incidence of hepatocellular neoplasms appears to be low in rats. Solleveld, Haseman & McConnell (1984) found the incidence of hepatocellular adenomas to be 8.6 and 2.8% in male and female inbred F344 rats, respectively. The incidence of hepatocellular carcinomas was 3% or less in

Table 8. Numbers of tumour-bearing male and female rats fed a diet containing BHT (excluding those with hepatocellular tumours)

Classification of tumour-bearing rats	BHT intake (mg/kg/day)...	Males				Females			
		0	25	100	250	0	25	100	250
	Effective no. of rats...	100	80	80	99	100	79	80	99
Total tumour-bearing rats		81	69	70	86	85	70	73	90
Rats with: malignant tumours		36	28	32	48	21	27	17	35
benign tumours		66	55	54	70	77	67	69	78
one tumour		38	43	42	37	38	23	30	36
two tumours		22	15	17	24	27	29	24	25
multiple tumours		21	11	11	25	20	18	19	29

Table 9. Occurrence of non-neoplastic lesions in rats fed a diet containing BHT

Site and lesion	BHT intake (mg/kg/day)... / Effective no. of rats...	No. of rats affected							
		Males				Females			
		0 / 100	25 / 80	100 / 80	250 / 99	0 / 100	25 / 79	100 / 80	250 / 99
Brain									
Gliosis		—	1	1	—	—	—	—	1
Malacia		1	—	—	—	—	—	—	—
Haemorrhage		—	2	—	—	1	1	—	—
Calcification		—	—	—	1	—	—	—	—
Kidney									
Nephropathy		47	39	45	36	57	33	33	36
Interstitial nephritis		4	1	1	3	3	1	1	3
Nephrocalcinosis		13	2	—	6	59	48	47	37
Cyst		5	4	—	9	3	—	1	10
Inflammation		19	13	13	24	28	18	9	30
Hydronephrosis		8	1	—	1	3	5	1	2
Haemorrhage		—	—	—	—	1	—	—	—
Urinary bladder									
Inflammation		20	12	11	17	13	9	5	14
Testis									
Atrophy		63	48	47	44				
Leydig-cell hyperplasia		3	1	—	2				
Inflammation		—	—	—	3				
Seminal gland									
Inflammation		3	4	5	4				
Ovary									
Angiectasis						1	—	—	—
Cyst						—	1	2	5
Uterus/vagina									
Inflammation						1	2	1	1
Cyst						9	4	5	8
Myometric hyperplasia						—	—	1	—
Heart									
Fibrosis		72	40	44	36	43	22	25	9
Endocardiosis		1	4	1	2	—	—	—	2
Calcification		—	—	2	—	2	1	1	3
Arteries									
Calcification		2	—	—	1	11	1	1	—
Arteriitis		16	19	27	22	14	6	8	2
Lung									
Adenomatosis		3	2	4	3	1	2	1	3
Fibrosis		—	—	—	2	1	—	—	3
Foamy cells		—	—	—	1	—	1	—	2
Calcification		—	—	—	—	2	—	—	—
Pituitary glands									
Angiectasis		1	—	—	—	1	3	2	3
Haemorrhage		1	—	2	—	2	—	3	1
Cyst		1	1	4	3	—	—	—	1
Thyroid gland									
C-cell hyperplasia		21	25	30	32	31	19	20	24
Dermoid cyst		1	—	—	—	—	—	—	—
Epithelial desquamation		—	—	2	1	1	—	—	—
Cystic follicles		—	—	—	1	—	—	—	1
Ultimobranchial body		—	—	—	—	—	—	1	—
Parathyroid gland									
Hypertrophy		3	1	3	1	6	—	1	—
Adrenal gland									
Fatty metamorphosis		4	6	7	9	2	1	4	5
Haemorrhage		1	1	2	—	13	10	6	16
Cortical hyperplasia		—	3	—	—	8	2	2	5
Medullary hyperplasia		3	—	—	—	2	—	—	—
Focal necrosis		—	—	1	—	3	—	1	1
Angiectasis		—	—	—	—	1	1	—	—
Atrophy		—	—	—	2	—	1	—	—
Cyst		—	—	—	—	1	3	1	1
Pancreas									
Inflammation		—	—	—	1	—	1	—	—
Acinar-cell atrophy		1	1	3	1	1	—	—	1
Islet-cell hyperplasia		6	8	5	10	5	1	5	5
Cyst		1	—	—	—	—	—	—	—
Acinar-cell hyperplasia		1	1	2	4	—	3	—	1
Fibrosis		—	—	—	—	1	—	—	—
Liver									
Fibrosis		—	—	1	—	1	4	1	3
Angiectasis		2	4	3	6	2	6	4	5
Eosinophilic necrosis		—	—	—	1	—	1	—	1

[*contd*]

Table 9—*continued*

Site and lesion	BHT intake (mg/kg/day)... / Effective no. of rats...	No. of rats affected							
		Males				Females			
		0 / 100	25 / 80	100 / 80	250 / 99	0 / 100	25 / 79	100 / 80	250 / 99
Basophilic areas		3	3	2	1	9	2	7	1
Focal cellular enlargement		6	7	14	8	1	7	11	16
Cysts		1	1	6	17	7	2	1	9
Fatty metamorphosis		11	10	3	3	3	3	1	—
Peliosis		2	2	4	4	1	2	4	—
Bile-duct proliferation		1	2	5	12	5	5	2	4
Haemorrhage		2	—	—	1	—	—	1	—
Stomach									
Erosion		—	—	1	1	1	—	—	—
Hyperkeratosis		1	1	—	1	—	—	—	—
Cyst		—	—	1	—	—	1	—	—
Small intestine									
Inflammation		—	1	—	—	—	—	1	—
Spleen									
Atrophy		—	1	—	—	—	—	—	—
Reticulosis		—	—	—	—	—	—	—	1
Thymus									
Cyst		—	—	—	—	—	1	—	1
Dermoid cyst		—	—	—	—	1	—	—	—
Lymph node									
Reticulosis		—	—	—	1	—	—	—	—
Plasmocytosis		—	—	—	—	1	—	—	—
Salivary gland									
Atrophy		1	4	—	1	—	1	—	—
Eye									
Cataract		—	—	—	—	1	—	—	1
Atrophy		—	—	—	—	—	1	—	—
Fibrosis		4	—	1	1	1	—	2	4
Lymphocytic infiltration		—	—	—	—	—	—	—	1
Mammary gland									
Cystic retention		5	4	2	1	22	15	18	15
Hyperplasia		1	1	1	—	14	8	5	6
Subcutaneous tissues									
Inflammation		—	—	—	—	—	1	—	—
Skin									
Erosion		4	—	3	—	—	2	1	1
Dermoid cyst		1	2	4	3	1	2	2	1
Inflammation		1	1	3	1	2	—	—	1
Bone									
Osteodystrophic fibrosis		—	—	1	—	6	1	2	1
Inflammation		—	1	—	2	—	—	—	—
Focal necrosis		—	—	—	—	—	—	—	1
Muscle									
Inflammation		2	1	2	—	—	—	—	—

both sexes (median lifespan, 50% survival age—28 months in both sexes). In another lifespan study using 320 outbred specified-pathogen-free Wistar rats of each sex (median life expectancy—33–36 months in males and 30–33 months in females), the incidence of hepatocellular adenomas was less than 3% in both sexes and only one heptocellular carcinoma occurred in a male rat during the third year of age (Deerberg, Rapp, Pittermann & Rehm, 1980). The same pattern was seen in our historical data for the same strain of Wistar rats fed a semi-synthetic diet for 132 wk (Olsen, Gry, Knudsen *et al.* 1985). These observations support the assumption that a better survival among controls in the present study would not have yielded a higher frequency of hepatocellular neoplasms in those groups. Pollard & Luckert (1979) found that liver tumours developed spontaneously in 87% of 132 germ-free Wistar rats above the age of 30 months. However, they could not exclude the possibility of contamination of the diet, which was prepared from vegetable components, with known hepatotoxic or oncogenic agents. *N*-Nitroso compounds, often reported as contaminants in laboratory-animal diets (Edwards, Fox, Policastro *et al.* 1979) were not found in the diet used in the present study.

Harman (1968) and Clapp, Satterfield & Bowles (1979) observed an increase in the mean lifespan of mice fed 0.5% BHT in a semi-synthetic diet or 0.75% BHT in a chow diet, but the maximum lifespan was not altered. In studies using chow diets and terminated within 25 months, male mice fed 0.6% BHT (National Cancer Institute, 1979) showed improved survival, but this effect was not mentioned for rats fed dietary levels of 1% BHT (Hirose *et al.* 1981) or 0.6% BHT (National Cancer Institute, 1979) nor for mice fed 0.5% BHT (Shirai *et al.* 1982). The better survival of the treated rats in our 33-month study may be connected with the reduced body weight, as indicated by Yu, Masoro, Murata *et al.* (1982).

Apart from the results of T. M. Brooks, P. P. Hunt, E. Thorpe & A. I. T. Walker (unpublished report, Shell Research Ltd, 1976), who found lung neoplasia

in BHT-treated mice, other long-term studies in rodents suggest that BHT is not carcinogenic (Hirose *et al.* 1981; National Cancer Institute, 1979; Shirai *et al.* 1982). In contrast, our study showed a dose-related and high incidence of benign and malignant hepatocellular neoplasia in both sexes, the males being the more susceptible. The reason for this discrepancy is most likely to be the duration of the study, as most of the neoplasia was found in animals at least 2.5 yr old. The metabolic breakdown of BHT in the liver by the cytochrome *P*-450 system was not determined in this study. A decrease in the function of cytochrome *P*-450 with ageing has been demonstrated in rats (McMartin, O'Connor, Fasco & Kaminsky, 1980). Furthermore the activity of the cytochrome *P*-450 system was lower in rats fed semi-purified diet than in rats fed chow diet (Marshall & McLean, 1971). Whether these mechanisms play a role in the development of hepatocellular neoplasms at a late stage in the life of rats given BHT needs to be elucidated.

Studies have shown that BHT inhibited liver-tumour formation when fed to rats before or concurrently with 2-acetylaminofluorene (Ulland *et al.* 1973). BHT also inhibited tumours of the forestomach in mice and of the mammary gland in rats treated with some polycyclic aromatic hydrocarbon carcinogens (Wattenberg, 1972). These effects may have been due to induction of detoxification pathways or to direct interaction with the carcinogenic compounds. On the other hand, BHT can also enhance the induction of liver tumours when given to rats after 2-acetylaminofluorene administration (Maeura & Williams, 1984; Peraino *et al.* 1977).

BHT shows properties similar to the well-known promoters of liver carcinogenicity, phenobarbital and trichlorodichlorophenylethane (DDT). However, on a molar basis, phenobarbital and DDT are several orders of magnitude more potent than BHT in inducing the liver-microsomal mixed-function oxidases (Peraino *et al.* 1977) and also in promoting liver carcinogenesis (Maeura & Williams, 1984; Peraino *et al.* 1977). Although phenobarbital has induced hepatocellular neoplasms when administered alone for long periods (IARC Working Group, 1982; Ward, 1983), no mutagenic effect has been demonstrated in *Salmonella typhimurium* (McCann, Choi, Yamasaki & Ames, 1975). Mutagenicity tests on BHT *in vitro* (Joner, 1977; Williams, Shimada, McQueen *et al.* 1984) and *in vivo* (Bruce & Heddle, 1979; Epstein & Shafner, 1968) do not indicate a direct DNA-damaging effect. Whether the hepatocellular neoplasms observed in this study were due to a carcinogenic effect of BHT *per se* or to an effect of BHT as a promoter of early spontaneous preneoplastic foci found in the liver of untreated rats (Schulte-Herman, Timmermann-Trosiener & Schuppler, 1983) remains uncertain.

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