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THREE-GENERATION TOXICITY STUDY OF RATS INGESTING BROWN HT IN THE DIET

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Abstract—Brown HT was fed to rats of both sexes over three generations at dietary concentrations designed to provide daily intakes of 0, 50, 250 and 500 mg Brown HT/kg body weight/day. During the study a number of females died or failed to nurse their litters. This was so severe following the first mating of F₁ adults that the animals were remated to provide the next generation. None of these effects were related to treatment. Body weight and food and water intakes were not adversely affected by treatment. No effects of treatment were seen on reproductive performance or foetal and pup development, apart from slight evidence of a treatment-related retarded ossification of the third sternebrae. Organ weights at autopsy showed two changes, one of which was increased kidney weights which, although not present in every generation, seemed to be related to treatment. The other, increased caecum weights, occurred in adult high-dose females of early generations, but not in males or later generations of the study. Apart from brown coloration of tissues, macroscopic and microscopic examination revealed no treatment-related changes. It was concluded that the no-untoward-effect level in the present study was 250 mg Brown HT/kg/day.

INTRODUCTION

The water-soluble azo dye Brown HT (CI (1971) No. 20285) is principally composed of the disodium salt of 4,4'-(2,4-dihydroxy-5-hydroxymethyl-1,3-phenylenebisazo)di(naphthalene-1-sulphonic acid). It is used for colouring a variety of food and drink products.

It is permitted for use in the UK under the Colouring Matter in Food Regulations 1973 (Statutory Instrument 1973, no. 1340), and under the Treaty of Accession to the EEC, the UK may continue to authorize its use under national legislation, pending a review of its toxicological status by the EEC authorities (1978).

Acute toxicity studies in rats and mice showed that the oral LD₅₀ exceeded 2 g/kg in both species, whereas the intraperitoneal LD₅₀ values for these species were estimated to be in the range 220–375 mg/kg (Hall *et al.* 1966).

Two short-term feeding studies in the rat (Chambers *et al.* 1966; Hall *et al.* 1966) established no-effect levels of 0.5 and 0.6% of the diet (approximately 250–350 mg Brown HT/kg/day), respectively. At higher dose levels (up to 2.0% of the diet), mild anaemia and slightly reduced body-weight gains were findings that may have been associated with treatment in both studies. Brown coloration of the Kupffer cells of the liver, the proximal convoluted tubules of the kidney and the lymph nodes was also observed at dose levels above 0.5%. Hall *et al.* (1966) reported a mild degree of renal dysfunction and significant increases in the relative weights of several organs, mainly at the 2% level, but these changes were not observed by Chambers *et al.* (1966). In a short-term feeding study in the pig, there were no adverse findings with doses up to 100 mg Brown HT/kg body weight/day (Hendy *et al.* 1978).

Long-term feeding studies in rats (Carpanini *et al.* 1978) and mice (Drake *et al.* 1978) failed to reveal any

carcinogenic effect of Brown HT at dose levels up to 500 and 700 mg/kg/day, respectively. The no-untoward-effect levels in these studies were 1.0% of the diet (*c.* 500 mg/kg/day) in the rat, and 0.1% (*c.* 140 mg/kg/day) in the mouse. In the latter study, the findings at the highest dietary level (0.5%) were a slightly reduced body-weight gain and a lower heart weight in males, reduced total leucocyte count and packed cell volume in females, and brown coloration of the lymph nodes and intestine in both sexes and of the ovaries and uterus.

In vitro genotoxicity assays have shown that Brown HT does not induce point or frameshift mutations in several strains of *Salmonella typhimurium* (Bonin & Baker, 1980; Haveland-Smith & Combes, 1980), and does not produce detectable DNA damage in repair-deficient strains of *Escherichia coli* (Haveland-Smith & Combes, 1980).

An early metabolic study (Fore *et al.* 1967) showed that Brown HT was decolorized, and a number of other azo dyes (Walker, 1970) undergo azo-reductive fission, when incubated with rat-liver homogenates. Recently Phillips *et al.* (1987) have reported the presence of both Brown HT and its azo-reduced metabolite, naphthionic acid, in the faeces of rats, mice and guinea-pigs, whereas naphthionic acid was the only major metabolite detected in urine. Studies by Mallett *et al.* (1982) demonstrated that the metabolites of Brown HT incubated in a continuous culture of rat hind-gut microflora were virtually identical to those isolated from rat faeces by thin-layer chromatography. The major metabolite was again naphthionic acid.

The present investigation was carried out on behalf of the UK Colours Steering Group to provide the data requested by the EEC Scientific Committee for Food (SCF, 1975), the UK Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment (FACC, 1979), and the Joint

FAO/WHO Expert Committee on Food Additives (JECFA, 1978).

The data contained within this paper were reviewed by the SCF in July 1983 and an ADI was established at 0.3 mg/kg body weight, with no request for further data. Similarly these data were reviewed in March 1984 by JECFA and an ADI of 0–1.5 mg/kg body weight was established.

MATERIALS AND METHODS

Test material. Brown HT was supplied by Williams (Hounslow) Ltd, Hounslow, Middlesex. Analysis carried out by the suppliers showed that the material contained: dye, 70.4%; subsidiary dyes, <15%; volatile matter, 2.1%; water-insoluble matter, 0.06%; sodium chloride, 31.3%; arsenic, <1 ppm; lead, <5 ppm; iron, 30 ppm; copper, <1 ppm; chromium, <1 ppm; zinc, 1 ppm; cadmium, <1 ppm; mercury, 0.8 ppm; free aromatic amines (as aniline), <10 ppm; ether extractable material, 0.1%; naphthionic acid, 0.7%. (Figures for impurities have been prorated to correspond to 100% dye content.)

Animal maintenance. Weanling rats of a Wistar-derived outbred strain were outlined as litters from a specified-pathogen-free breeding colony (University of Nottingham, Joint Animal Breeding Unit, Loughborough, Leicestershire). Groups of six of the same sex and treatment were housed in polypropylene cages with open-mesh stainless-steel wire tops and floors (North Kent Plastic Cages Ltd, Dartford, Kent), suspended on racks over high-wet-strength paper for collection of excreta. Pregnant females were housed in similar cages with solid floors and sawdust bedding. Throughout the study the animals were maintained in air-conditioned rooms with an ambient air temperature of approximately 20°C, relative humidity in the range 40–60%, and an automatic 14/10-hr light/dark cycle. The weanling rats were allowed to acclimatize to the environmental conditions for 2 wk before receiving the test diets.

Diets. The animals were allowed free access to food and tap-water during all phases of the study. Control rats received a basic powdered diet, Spratt's Laboratory Animal Diet No. 2 (Spratt's Patent Ltd, Barking, Essex). Test diets contained concentrations of Brown HT calculated on the basis of food intake and body weight measurements to provide, as accurately as possible, a constant intake of 0 (control), 50, 250 or 500 mg Brown HT/kg body weight/day throughout the experiment. When rats of more than one age shared the same cage the dietary concentration was calculated on the basis of the youngest animal's food intake and body weight. A sample of each diet was analysed for Brown HT content prior to use.

Experimental design and conduct

The scheme of the study is shown in Fig. 1. Weanling F_0 rats were randomized into four groups for each sex and fed diets containing sufficient Brown HT to provide daily intakes of 0 (control), 50, 250 or 500 mg/kg body weight. The six test groups each contained 36 rats and the untreated control groups 60 rats each. Mating of the F_0 animals, to provide F_1 litters, commenced after 60 days on the test diet. Pairs were selected from within each group by random

techniques, with changes where necessary to avoid sibling matings. The first 24 females from the control group and 12 females from each of the three treatment groups to conceive were time-mated so that the date of coition was known (day of mating was designated day 0). These females were killed on day 20 of gestation for a gross autopsy and a detailed examination of the uterus and its contents. The foetuses were examined for external and visceral defects, weighed and preserved for examination of skeletal development. A gross post-mortem examination was performed on the males used for the timed matings. The remaining males and females were housed in pairs for 12 days (approximately three oestrous cycles) to allow mating. The males were then removed and the pregnant females were individually housed in solid-floored cages and allowed to deliver and rear their young. Survival, growth and development of the offspring were monitored over the first 21 days of life. After weaning, young males and females were randomly selected from the litters, either to constitute the next generation (F_1) or for autopsy. Where possible, at least one male and one female were selected from each litter to provide equal numbers for the F_1 generation as were used for the F_0 generation. Distribution was such that the F_1 mean weights were similar for each group. At the same time, where possible, one male and one female were chosen from each litter and killed at 33 ± 2 days after birth and a full autopsy was carried out. A gross autopsy was performed on the remaining young rats. The F_0 adult animals (random-mated) were killed and subjected to a full post-mortem examination.

The procedure described for the F_0 generation was followed with the F_1 rats to provide the F_{2a} litters. However, because of low litter survival, the F_1 adults were mated a second time using the same pairs to provide the F_{2b} litters. After weaning, the F_{2a} and F_{2b} young were selected either to continue treatment or for autopsy as described for the F_1 generation. The group sizes of the F_{2a} generation were: control, 36 per sex; test, 24 per sex. These animals continued on treatment for 42 days, after which they were killed and given a full post-mortem examination. The group sizes of the F_{2b} generation were the same as those used for the F_0 and F_1 generations. The procedure described for the F_0 generation was repeated with the F_{2b} rats to provide the F_3 litters. After weaning, up to three males and three females were selected at random from each litter for a full post-mortem examination, performed on day 70 ± 2 *post partum*. The remaining F_3 young were killed for a gross autopsy. A histological examination was carried out on the tissues taken from the F_3 control rats and those given 500 mg Brown HT/kg/day.

Throughout the study mating was permitted only between males and females from the same generation and treatment group, and offspring received from weaning the same test or control diet as their parents.

Observations. In each generation (F_0 , F_1 and F_{2b}) individual body weights were recorded at weekly intervals and measurements of food and water intake were made at twice-weekly intervals for a period of approximately 60 days prior to mating. In the F_{2a} and F_3 generations these parameters were monitored for 42 and 49 days, respectively. From these measure-

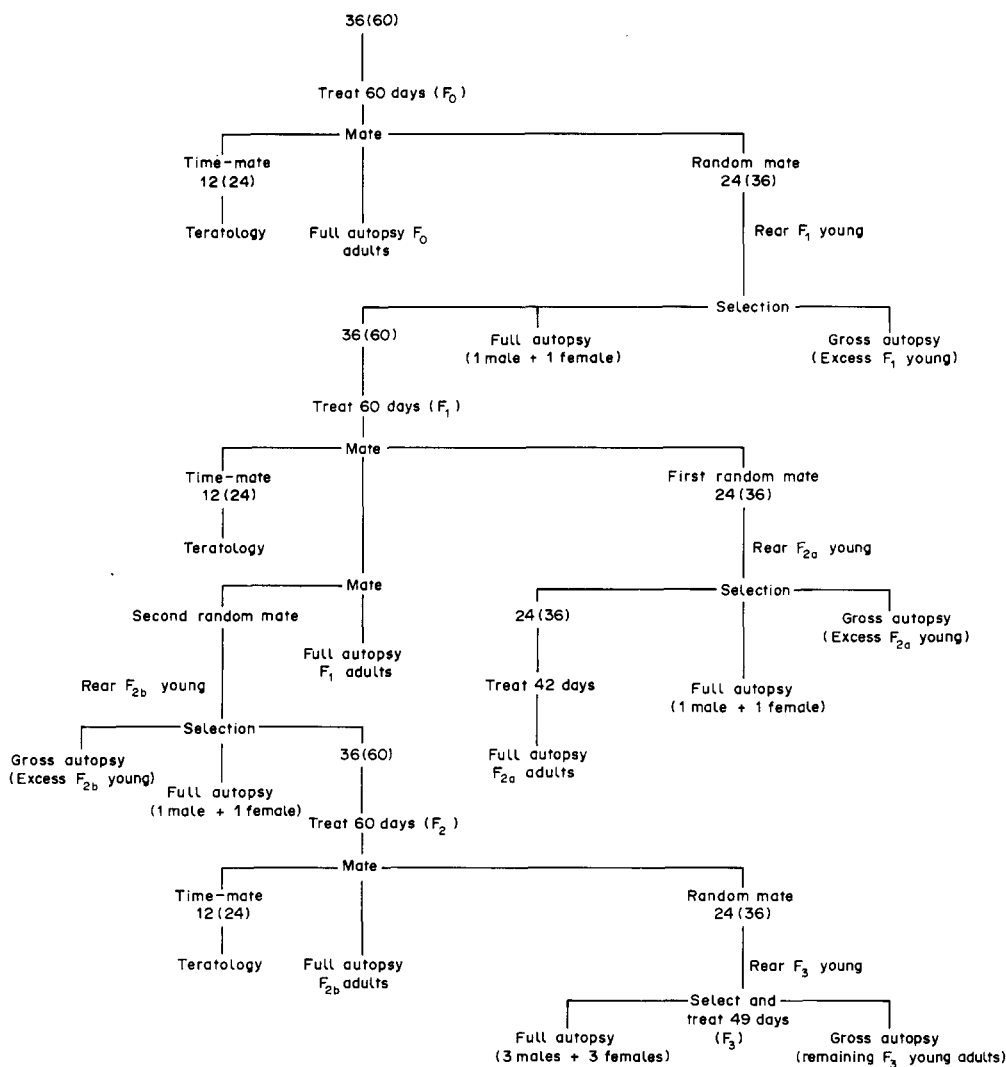


Fig. 1. Scheme of the multigeneration reproduction study. The numbers of rats of each sex for each treatment group are given with the numbers of control animals in parentheses.

ments the average daily food and water consumption and colouring intake per rat were calculated. All animals at all stages of the study were observed daily, and any considered unfit to continue were killed and a full autopsy was performed.

Teratology. Time-mated females were killed by cervical dislocation on day 20 of pregnancy. The number of corpora lutea in each ovary was recorded together with the number and position of foetuses and resorptions in each uterine horn, foetal viability and the presence of any visible foetal malformations. The resorptions were classified as early or late depending on the presence or absence of visible foetal tissue. Each viable foetus was weighed, preserved in 95% alcohol and identified according to position in the uterine horn. After fixation in alcohol, the foetuses were eviscerated, the eyes and brown fat overlying the scapulae were removed, and the sex was determined. The ossified parts of the eviscerated

foetuses were stained with Alizarin Red S by a method based on that described by Staples & Schnell (1964), and examined for skeletal abnormalities and variations in the degree of ossification.

Physical and neurological development of litters. Dams and their litters were inspected daily for the first 21 days of life. The date of parturition was recorded and litter survival was calculated as the percentage of live pups born that survived to day 7, 14 and 21 after birth. Litters were weighed on day 7, 14 and 21 and the sex of the pups was verified. Several indicators of physical and neurological development were also monitored during the first 21 days of life. The tests used and the criteria for a positive observation of physical development were as follows: eye opening, one or both eyes fully opened; ears uncurled, one or both pinna fully unfolded; tooth eruption, one or more incisors visible above the gums; hair growth, a coat of fine fur covering the

body. The tests used and criteria for a positive observation of auditory and neuromuscular development were:

Righting response: to test the mid-air righting reflex, each animal was dropped from a supine position at a height of 30 cm onto a padded surface. A stable landing on all four legs was considered a positive response.

Startle response: each animal was isolated and subjected to a single metallic 'click' generated at a distance of 20 cm. Any sudden movement was considered a positive response.

Clinging ability: each animal was supported on the palm of the hand and allowed to touch a thin wire suspended 30 cm above a bench surface. Grasping usually ensued, immediately followed by attempts to flex the forelimbs and support the body with the hindlimbs. Grasping the wire with one or both hindlimbs was regarded as a positive response.

For each litter, the day of appearance of a particular developmental milestone was taken as the number of days after birth when a positive observation or response was obtained for all pups in the litter.

Post-mortem examination. Animals subjected to a full post-mortem examination (see experimental design) were weighed following an overnight fast (with free access to water) and then killed by exsanguination whilst anaesthetized by an intraperitoneal injection of sodium pentobarbitone. During the autopsy, the adrenal glands, brain, caecum (with and without its contents) gonads, heart, kidneys, liver, spleen, stomach and thyroid gland were removed and weighed (the thyroid gland was not weighed during autopsy of the F_{2b} and F_3 generations). These organs, together with samples of the aorta, colon, eye, Harderian gland, lungs, lymph nodes (cervical and mesenteric), mammary tissue, oesophagus, pancreas, rectum, salivary glands, skeletal muscle, skin, small intestine, spinal cord, thymus, trachea, urinary bladder, vena cava, seminal vesicles and epididymis or uterus and vagina, and any other tissues that appeared to be abnormal, were preserved in 10% neutral buffered formalin.

Animals subjected to a gross autopsy (see experimental design) were allowed free access to food and water until they were killed. Young rats were killed by inhalation of either diethyl ether or carbon dioxide. Older animals were killed by exsanguination under barbiturate anaesthesia. A general examination of all tissues listed above was carried out. Any tissues that were considered to be abnormal were preserved in 10% buffered formalin.

Histopathology. Paraffin wax sections of the tissues taken at autopsy from the F_3 generation control rats and rats given 500 mg Brown HT/kg body weight were stained with haematoxylin and eosin for examination by light microscopy.

Statistical evaluation

Histopathological findings (F_3 generation) were analysed by the Fisher's exact probability test (Armitage, 1980). Full or gross post-mortem observations and the number of dams that produced litters/number of dams mated were analysed for a positive or negative dose-related trend by the method

of Peto *et al.* (1980). Organ weights were analysed using an analysis of variance and Student's *t* test.

For the analysis of observations recorded during the examination of alizarin-stained foetuses (F_3 generation) the litter was considered to be the basic experimental unit. For each skeletal element, the proportion of foetuses within each litter exhibiting the normal feature (the observation that occurred most frequently in all foetuses from the control group) was determined. The proportions were subjected to arcsin transformation (Snedecor & Cochran, 1968) followed by one-way analysis of variance.

Other observations were analysed by analysis of variance and a least significant difference test (Snedecor & Cochran, 1968). The level of significance chosen in all cases was $P < 0.05$.

RESULTS

Although the health of the animals was generally good throughout the study several deaths occurred. These were confined to females at the time of parturition, mainly in F_1 generation animals (Table 1). In addition a number of females from both F_1 and F_{2b} generations failed to nurse their litters, resulting in pup deaths. The occurrence of these changes was not related to treatment. The loss of litters following the first mating of the F_1 generation was so great that insufficient pups survived for a full continuation of the study. The F_1 animals that survived were remated and despite the recurrence of the signs of ill-health in a number of pregnant females, a sufficient number of young and range of litters survived to allow the study to be continued.

There were occasional differences between treated and control groups, but overall there were no adverse treatment-related trends in either food or water consumption or in body-weight gain. Calculation of Brown HT intake, based upon body weight and food-intake data, indicated that the dose received by the treated groups was within 10% of that intended.

Reproduction performance

The proportion of paired females that became pregnant (fertility rate) was similar in the control and

Table 1. Summary of deaths and litter losses for female rats of generations F_1 and F_{2b} created with Brown HT

Parameter	No. at doses (mg/kg/day) of:			
	0	50	250	500
F_1 generation (first mating)				
Females pregnant	29	21	20	20
Female deaths	4	0	2	0
Litters lost	13	12	13	9
F_1 generation (second mating)				
Females pregnant	27	22	16	21
Female deaths	1	2	0	0
Litters lost	8	8	8	6
F_{2b} generation				
Females pregnant	34	24	19	22
Female deaths	0	0	0	0
Litters lost	9	6	3	5

The litter losses occurred between parturition and day 4. Most female deaths occurred at parturition or shortly after. No female deaths or litter losses occurred in the F_0 generation.

Table 2. Summary of data on the fertility of dams and on the survival and growth of their young during the first 21 days *post partum* in a three-generation study of rats fed Brown HT at dose levels of up to 500 mg/kg/day

Treatment group (mg/kg/day)	Dams			Pups			
	No. mated†	Total no. of litters	No. of surviving litters†	No. alive		Mean pup weight (g)	
				Day 0	Day 7	Day 7	Day 21
F₀ generation							
0	36	30	30	9.7 ± 1.9	8.5 ± 2.6	14.7 ± 2.4	44.3 ± 5.5
50	24	20	20	10.9 ± 2.2	10.5 ± 1.9*	14.8 ± 1.3	42.1 ± 4.4
250	24	24	24	10.1 ± 1.8	9.3 ± 2.2	14.9 ± 2.3	45.0 ± 4.7
500	24	20	20	10.4 ± 2.1	9.1 ± 2.2	15.1 ± 1.5	45.9 ± 4.8
F₁ generation (first mating)							
0	36	29	16	8.9 ± 1.7	7.9 ± 2.6	13.7 ± 3.8	45.2 ± 7.6
50	24	21	9	9.3 ± 1.4	8.9 ± 1.3	14.7 ± 1.8	45.7 ± 2.9
250	24	20	7	9.0 ± 2.8	8.4 ± 2.6	14.5 ± 2.8	47.0 ± 7.7
500	24	20	11	10.2 ± 2.4	9.2 ± 3.3	12.8 ± 1.9	43.5 ± 4.5
F₁ generation (second mating)							
0	30	27	19	8.6 ± 2.5	7.5 ± 2.8	15.6 ± 5.6(18)	51.0 ± 8.9
50	23	22	14	10.0 ± 1.5	9.3 ± 2.4	15.5 ± 2.4(13)	48.0 ± 6.4
250	21	16	8	9.1 ± 2.5	8.9 ± 2.5	15.6 ± 1.8(7)	48.7 ± 4.8
500	23	21	13	9.1 ± 3.3	8.9 ± 3.1	15.8 ± 1.5(12)	48.9 ± 5.9
F_{2b} generation							
0	36	34	25	10.0 ± 2.1	8.6 ± 2.5(24)	3.8 ± 2.1	45.4 ± 5.0(24)
50	24	24	18	10.7 ± 2.1	9.0 ± 2.8	14.1 ± 2.1	46.5 ± 4.7
250	21	19	16	10.3 ± 1.6	9.6 ± 1.8	14.6 ± 1.6	47.1 ± 4.1
500	24	22	17	9.8 ± 2.9	7.6 ± 3.0	13.1 ± 3.6	46.6 ± 7.5

†Data were analysed using a test (Peto *et al.* 1980) for dose-related trend.

The values for pups are expressed as means ± SD for the number of viable litters shown (either in column 4 or, if fewer, in parentheses). Values marked with an asterisk differ from those of controls ($P < 0.05$) by a least significant difference test, subsequent to a significant result from analysis of variance over all treatments.

treated groups (Table 2), and the number of non-viable litters did not increase with treatment.

The total numbers of litters lost through parental neglect or death of the female during the reproductive phase of the F₀, F₁ and F_{2b} generations are shown in Table 1. The summary table shows that the litter losses occurred in all groups and that they were not related to treatment with Brown HT.

Pup survival, growth and development

Although litter survival was reduced in all groups during the first few days of life, there were no statistically significant differences between the control and treated groups in average litter size, or growth and development of the young (Table 2) or in the sex ratios. Apart from a few young that failed to gain weight at a rate comparable with their litter mates, the general condition of the viable litters was good throughout lactation and weaning.

Teratology

The autopsy findings of the uterine contents of the time-mated females killed on day 20 of gestation are summarized in Table 3. In the F₀ generation there were significantly fewer corpora lutea in the high-dose group than in the control, and the average numbers of implantations and live foetuses were also slightly below control values in this group. Conversely, mean foetal weight was significantly higher in this group and in the low-dose group than in the control. Other autopsy findings of the F₀ generation were of similar incidence in the control and treated females. Apart from a lower ratio of post-implantation losses to implantations in the 50-mg/kg group compared with the control in the F_{2b} generation, there were no significant differences in any of

the variables studied as indicators of uterine development in the F₁ and F_{2b} generations.

There were no external, visceral or skeletal abnormalities in the foetuses (F₃ generation) and only minor variations in the degree of skeletal ossification. A statistical analysis of the findings in the alizarin-stained preparations revealed some slight evidence of retarded ossification of the third sternbrae with treatment.

Post-mortem and histopathology findings

In both sexes there were a number of statistically significant differences between the treated and control groups in the weights of the various organs taken at autopsy of adult (Table 4) and young (Table 5) rats. In many instances these differences were no longer statistically significant when the organ weights were expressed relative to body weight. The few organ-weight differences that were statistically significant were isolated and showed no dose-related trend.

The only finding that was consistently associated with treatment at the adult stage of each generation was a statistically significant increase in kidney weight. This was apparent at the 500-mg/kg dose level of male rats in all generations; in the male and female rats at the 250-mg/kg level in the F₀ generation, in female rats at the 50-mg/kg level in the F_{2b} generation, and in the female top-dose group in the F₁ and F₂ generations. These differences remained statistically significant when the kidney weight was expressed relative to body weight in all cases except for the 50-mg/kg group in the F_{2b} generation and the female top-dose group in the F₁ generation. There was a statistically significant increase in relative kidney weight in young top-dose level rats of both sexes

Table 3. Summary of the uterine contents of female rats fed Brown HT at dose levels up to 500 mg/kg/day throughout pregnancy

Treatment group (mg/kg/day)	No. of pregnant females examined	No. of corpora lutea	No. of implantations	Pre-implantation losses	Post-implantation losses	No. of live foetuses	Litter weight (g)	Mean foetal weight (g)
0	23	12.3 ± 2.1	11.5 ± 1.8	0.8 ± 1.3	1.0 ± 2.1	10.5 ± 3.2	31.2 ± 6.3(22)	2.8 ± 0.2
50	10	11.2 ± 0.8	11.1 ± 0.9	0.1 ± 0.3	0.5 ± 0.7	10.6 ± 1.2	32.0 ± 4.3	3.0 ± 0.1*
250	11	11.5 ± 1.3	11.5 ± 1.3	0.0 ± 0.0	0.6 ± 1.0	10.8 ± 1.8	32.3 ± 4.8	3.0 ± 0.2
500	11	10.4 ± 2.2*	9.6 ± 3.3	0.8 ± 1.6	0.3 ± 0.5	9.3 ± 3.2	27.8 ± 8.9	3.1 ± 0.4*
F₀ generation								
0	20	11.0 ± 2.5	10.8 ± 2.5	0.2 ± 0.6	0.6 ± 1.0	10.2 ± 2.4	29.1 ± 6.6	2.9 ± 0.2
50	11	11.1 ± 1.5	10.7 ± 1.7	0.4 ± 0.8	0.3 ± 0.5	10.5 ± 1.8	31.3 ± 6.0	3.0 ± 0.2
250	10	11.8 ± 1.6	11.8 ± 1.6	0.0 ± 0.0	0.6 ± 0.5	11.2 ± 1.8	37.6 ± 5.5	3.0 ± 0.2
500	11	11.4 ± 1.1	10.7 ± 1.2	0.6 ± 1.1	0.6 ± 1.5	10.1 ± 1.6	30.4 ± 5.2	3.0 ± 0.1
F₁ generation								
0	24	13.2 ± 1.9	12.2 ± 1.9	1.0 ± 1.3	1.3 ± 1.7	10.9 ± 2.6	33.1 ± 7.8	3.0 ± 0.2
50	11	12.6 ± 1.3	12.2 ± 1.5	0.4 ± 0.7	0.3 ± 0.5	11.9 ± 1.5	36.0 ± 3.6	3.0 ± 0.1
250	12	13.0 ± 1.1	12.8 ± 1.0	0.2 ± 0.4	0.9 ± 0.5	11.9 ± 1.1	35.9 ± 3.2	3.0 ± 0.1
500	11	13.0 ± 1.7	12.4 ± 1.4	0.6 ± 0.8	0.6 ± 0.8	11.7 ± 1.4	35.3 ± 4.1	3.0 ± 0.2
F_{2b} generation								

Each post-mortem examination was carried out on day 20 of pregnancy. Values are expressed as means ± SD for the number of females shown (either in column 2, or if fewer, in parentheses). Values marked with an asterisk differ from those for controls ($P < 0.05$) by a least significant difference test subsequent to a significant result from an analysis of variance over all treatments.

Table 4. Summary of statistically significant differences in organ weights between control and Brown HT-treated groups of adult rats of generations F₀, F₁, F_{2a} and F_{2b}

Organ	Dose level (mg/kg)...	Generations with organ weights less than those of the control†						Generations with organ weights greater than those of the control‡										
		Male			Female			Male			Female							
		50	250	500	50	250	500	50	250	500	50	250	500					
Brain																		
Heart																		
Liver																		
Spleen																		
Kidneys																		
Stomach																		
Full caecum																		
Empty caecum																		
Adrenals																		
Gonads																		
Thyroids§																		

†The numbers denote the generation (F₀ = 0; F₁ = 1; F_{2a} = 2; F_{2b} = 3) and the letters A and R refer to absolute and relative weights, respectively. Numbers with an asterisk denote differences in both absolute and relative weights of an organ.

‡The body weights of the 50-mg/kg dose group in the F_{2b} generation were high, so the organ-weight differences were only significant when expressed as absolute weights.

§No data for F_{2b} and F₃ generations.

Table 5. Summary of statistically significant differences in organ weights between control and Brown HT-treated groups of young rats of generations F₁, F_{2a}, F_{2b} and F₃

Organ	Dose level (mg/kg)...	Generations with organ weights less than those of the control†				Generations with organ weights greater than those of the control†			
		Male		Female		Male		Female	
		50	250	500	500	50	250	500	500
Brain									
Heart									
Liver									
Spleen									
Kidneys									
Stomach									
Full caecum									
Empty caecum									
Adrenals									
Gonads									

†The numbers denote the generation (F₁ = 1; F_{2a} = 2; F_{2b} = 3; F₃ = 4) and the letters A and R indicate absolute or relative weights, respectively. Numbers with an asterisk denote differences in both absolute and relative weights of an organ. Autopsies were carried out at 33 ± 2 days *post partum* for F₁ and F_{2a} generations, and at 70 ± 2 days *post partum* for F_{2b} and F₃ generations.

of the F_{2b} generation and in the males of the top-dose level in the F₃ generation.

Caecum weights of adult treated females of the F₀ and F₁ generations were often greater than those of the controls. Males showed no such differences but young females showed a similar trend in the final generation. Remaining differences in organ weights were isolated and showed no dose relationship.

Macroscopic findings at autopsy were similar for all groups, but the mesenteric lymph nodes of treated animals were frequently reported to be brown, with the greatest coloration at the highest dose. Additionally, brown coloration of the caecum and molars was recorded for some of the highest-dose animals.

Microscopic examination of tissues from F₃ rats revealed no changes that were consistently associated with treatment.

DISCUSSION

It is possible that the deaths of F₁ females at parturition and other signs of ill-health were due to rat corona virus, as characteristic lesions were identified in the lungs and Harderian glands of F₁ females at autopsy. Serological analysis failed to confirm this diagnosis and a positive identification of the causative agent was not possible. It is considered likely that the stress of littering may have aggravated an otherwise mild, subclinical infection.

The lack of effect of treatment on body weight and food or water intakes are in agreement with the results of Carpanini *et al.* (1978).

While brown coloration of the gastro-intestinal tract and mesenteric lymph nodes has been reported in many earlier studies (Chambers *et al.* 1966; Drake *et al.* 1978; Grant & Gaunt, 1987; Hall *et al.* 1966; Hendy *et al.* 1978) there is no evidence that this has any adverse effect on animals. In a recent metabolic study using ¹⁴C-labelled Brown HT (Phillips *et al.* 1987), it was shown that small amounts of the compound and/or its metabolites accumulate in most tissues of the rat during repeated daily administration, but the accumulation was only tissue-specific in the mesenteric lymph nodes and kidney. An increase in the accumulation of colouring with prolonged treatment is also suggested in the study reported here. The incidence of coloration of the tissues was greatest in the adult animals exposed to the highest dose of Brown HT, and most infrequent in the young killed shortly after weaning.

The slight caecal enlargement seen on several occasions in treated females may represent a mild effect of treatment. A similar time-related enlargement of the caecum has been observed in rats fed diets containing other food colourings including Sunset Yellow FCF (Gaunt *et al.* 1967), Ponceau 4R (Brantom *et al.* 1987) and Green S (Moorhouse *et al.* 1987), and there is some evidence that the effect may be more pronounced in females (Moorhouse *et al.* 1987). The aetiology of caecal enlargement is uncertain, but it can be induced by the administration of a wide variety of compounds (Leegwater *et al.* 1974). It is generally considered to be a process of physiological adaptation and of no toxicological significance (Butterworth *et al.* 1975; Leegwater *et al.* 1974).

The kidney weights of rats given the highest dose

of Brown HT were consistently higher than those of the controls. An association with treatment is reinforced by the observation that these increases occurred only after a period of feeding and not in animals killed soon after weaning. In short-term feeding studies, Chambers *et al.* (1966) and Hall *et al.* (1966) found no significant change in the kidney weights of rats given approximately 500 mg Brown HT/kg body weight for 13 wk, but the latter group did find evidence of mild renal dysfunction (increased urinary glutamic-oxaloacetic transaminase activity) in both sexes at this dose level, and both studies reported significant increases in kidney weight at the highest dose level used (approximately 1000 mg Brown HT/kg body weight). Carpanini *et al.* (1978) also reported an increase in the relative kidney weights of male rats fed approximately 500 mg Brown HT/kg body weight for 2 yr.

In a recent metabolic study (Phillips *et al.* 1987) it was shown that up to 20% of the radioactivity associated with a single, oral dose of ¹⁴C-labelled Brown HT was excreted in the urine, mainly (80%) in the form of naphthionic acid, and that most (70%) of this renal clearance was effected within 24 hr of dosing. From these findings, it is reasonable to assume that in feeding studies, where treatment is continuous, there is a continuous excretory load on the kidney. The slight increase in kidney weight may be simply an adaptive response to this increased excretory load, or may indicate a toxic effect of treatment. From our findings it was not possible to determine the relative contribution of these factors to the changes seen. The fact that no renal damage was detected on histological examination is strong evidence against nephrotoxicity. However, as Kluge (1981) has pointed out, the absence of histological changes does not necessarily preclude injury. Brown HT and/or its metabolites have been shown to accumulate in the kidney (Chambers *et al.* 1966; Hall *et al.* 1966; Phillips *et al.* 1987) and, although rapidly cleared upon cessation of treatment (Phillips *et al.* 1987), may exert a mild toxic effect while present.

In the study reported here there was no evidence of an effect on the kidney at the intermediate dose level (250 mg Brown HT/kg body weight), and even at the highest treatment level the increases in kidney weight were slight and not always statistically significant in both sexes in each generation.

There was no evidence that Brown HT had any adverse effects on male fertility, on ovulation (as indicated by the number of corpora lutea), on the number of ova that implanted or on the number of implantations that gave rise to viable foetuses. These findings confirm and extend work by Grant & Gaunt (1987), which showed that there were no embryotoxic or teratogenic effects following the administration of Brown HT to female rats throughout pregnancy. The smaller number of corpora lutea, and slightly lower implantation rate in the F₀ high-dose females, suggested a possible effect on ovulation. However, as these changes were not encountered at the three later matings, it is considered unlikely that they were the result of treatment.

Although no major changes in skeletal development were encountered in this study, the finding of a slight dose-related reduction in ossification of the

third sternbrae is at variance with the observations of Grant & Gaunt (1987). They concluded that both the sternum and forelimbs of foetuses taken from females given 1000 mg Brown HT/kg body weight throughout pregnancy were at a slightly more advanced stage of development than those of the control. However, minor differences in the degree of skeletal ossification, such as those encountered in our study and in the previous teratology study (Grant & Gaunt, 1987), are often seen in 20-day rat foetuses (Aliverti *et al.* 1979), and in the absence of clear signs of embryotoxicity or retarded foetal development are considered acceptable variations of normal (Kimmel & Wilson, 1973).

The increased incidence of post-partum deaths during the first few days of the F₂ and F₃ generations was due to a lack of maternal care by unhealthy dams rather than to treatment. This view is based on the fact that in the generations in which females showed signs of ill-health at parturition, complete litter losses (i.e. litters in which all the young died within a few days of birth) were similar in control and treated groups. In addition, the litter survival rate to weaning was the same in the control and treated groups at all phases of the study.

In summary, the only findings in the study that could be related to treatment were caecal enlargement, brown coloration of the lymph nodes and some areas of the gastro-intestinal tract, and indications of nephrotoxicity in the form of increased kidney weight. There was no evidence that any of these findings had any adverse effects on the animals. However, on the basis of the changes in kidney weight, it is concluded that the no-untoward-effect level in this study is 250 mg Brown HT/kg/day.

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