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Research Section

CARCINOGENICITY STUDY OF AMMONIA-PROCESS CARAMEL IN F344 RATS*

A. MAEKAWA, T. OGIU, C. MATSUOKA, H. ONODERA, K. FURUTA, H. TANIGAWA, Y. HAYASHI and the late S. ODASHIMA

Division of Pathology, Biological Safety Research Center, National Institute of Hygienic Sciences, Kamiyooga 1-18-1, Setagaya-ku, Tokyo 158, Japan

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Abstract—The carcinogenicity of ammonia-process caramel, a food colouring, was examined in F344 rats. Caramel was dissolved in distilled water at levels of 0, 1 and 4% and groups of 50 male and 50 female rats were given 20–25 ml of one of these solutions/rat/day as their drinking water for 2 yr. There were no significant differences between the total incidences of tumours or mean survival times of control and experimental groups. A variety of tumours developed in all groups including the control group, and no dose-related effects were found either in the incidence or induction time of tumours in the various organs and tissues except in the pituitary gland of males, in which the incidence of tumours in males given 4% caramel solution was significantly higher than that in controls. Pituitary tumours are among the most common spontaneous tumours in ageing rats of this strain and have a variable incidence. In addition, almost all pituitary tumours detected in males given the 4% solution were microscopic tumours, and there was no significant difference between controls and treated groups in the incidence of hyperplasia or pre-neoplastic lesions in the pituitary gland. These results indicate that the significantly higher incidence of pituitary tumours in males given the 4% caramel solution was not related to caramel administration, but could be explained by the variability of the incidence of spontaneous pituitary tumours. Thus it is concluded that under these experimental conditions ammonia-process caramel was not carcinogenic in F344 rats.

INTRODUCTION

Ammonia-process caramel is widely used in Japan as a food colouring in sauce, soya sauce, luxury drinks and other foods and its total consumption in Japan is about 1500–1700 tonne/yr.

In recent Japanese studies ammonia-process caramel showed weak mutagenic activity in the Ames test with *Salmonella typhimurium* TA100 and weak activity in the chromosome-aberration test with Chinese hamster cells, but it showed no mutagenic effect in the Ames test with strain TA98, in the rec-assay or in the sister-chromatid exchange test (Kawachi, Komatsu, Kada *et al.* 1980).

In the present study we examined the carcinogenicity of ammonia-process caramel in F344 rats. This study was carried out as one of several co-operative studies in Japan to test mutagenic environmental chemicals for carcinogenicity in laboratory rodents (Odashima, 1980).

EXPERIMENTAL

Subchronic toxicity study

Specific pathogen-free Fischer (F344) rats of both sexes (5-wk-old) were purchased from Charles River Japan Inc. (Kanagawa). Rats were housed four to a plastic cage and kept in an air-conditioned animal room (temperature 25 ± 2°C, humidity 55 ± 10%). Seven-wk-old animals were divided into six groups, each consisting of ten males and ten females. Ammonia-process caramel (commercial sample) was obtained from Japan Caramel Industry Association (Tokyo), and dissolved in distilled water at concentrations of 0, 1.25, 2.5, 5, 10 and 20%. Rats were given 20 ml of one of these solutions/rat/day as their drinking water for 13 wk. Throughout the experimental period, rats in each group were given a basal diet (CRF-1, Charles River Japan Inc.) *ad lib.*

During the experimental period, all animals were observed daily and clinical signs were recorded. Body weights were measured every other week and haematological examinations were performed every 4 wk in control and 20% groups. At the end of the study, all survivors were killed for gross and microscopic examination. This study was used to determine appropriate dose-levels for the carcinogenicity study.

Carcinogenicity study

Rats. Five-wk-old specific pathogen-free Fischer (F344) rats of both sexes, purchased from Charles River Japan Inc., were maintained on basal diet (CRF-1, Charles River Japan Inc.) and tap water until they were 7 wk-old when the study was started.

Housing and feeding conditions. Rats were housed four males or five females to a plastic cage and kept in an air-conditioned animal room as in the subchronic toxicity study. The basic diet used in this study was tested for contaminants twice a year; no contaminants, such as pesticides, heavy metals, benzo[*a*]pyrene or aflatoxin, were detected.

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Caramel. The ammonia-process caramel used was the same class of commercial sample as used in the subchronic toxicity study. The specifications of this caramel were as follows (these specifications are the average values of four batches of this caramel used in this study): absorbance E 610 nm, 0.097; pH, 3.9; loss on drying, 41.2%; total nitrogen, 0.88%; ammonia nitrogen, 0.04%, 4-methylimidazole, <20 ppm; sulphur dioxide, not detectable; phosphate, not detectable; heavy metals, <10 ppm; arsenic, <1 ppm.

Experimental design. Animals were randomly divided into three groups, each consisting of 50 males and 50 females. Ammonia-process caramel was dissolved in distilled water at levels of 0 (control), 1 and 4%. Rats were given 20–25 ml of these solutions/rat/day as their drinking water for 2 yr. Caramel solutions were freshly prepared 3 times a week and the amount of solution consumed was measured to calculate the caramel intake. For investigation of the time-related effects of caramel, satellite groups of the control and 4% groups each consisting of 15 males and 15 females were set up. Throughout the experimental period, rats in all groups were given basal diet *ad lib.*

During the experimental period, all animals were observed daily, and clinical signs and mortality were recorded. Body weights were measured once a week during the first 13 wk of the study and then every 4 wk. Administration of caramel was stopped in wk 104 and thereafter tap water was given to animals in all groups, and observation was continued until wk 113 when all survivors were killed. Moribund or dead animals were autopsied completely and examined pathologically for the development of tumours in the following organs and tissues: brain, pituitary, thyroid (including parathyroid), thymus, lungs (including trachea), heart, salivary glands, liver, spleen, kidneys, adrenals, tongue, oesophagus, stomach, duodenum, jejunum, ileum, caecum, colon, rectum, urinary bladder, lymph nodes, pancreas, gonads, accessory genital organs, mammary gland, skin, musculature, peripheral nerve, spinal cord, sternum, femur, eyes, ear duct and nasal cavity. In the satellite groups, five males and five females were killed in wk 26, 52 and 78 for histological and haematological examination. Organs and/or tissues including tumour masses were fixed with buffered 10% formalin, and sections were routinely stained with haematoxylin and eosin.

RESULTS

Subchronic toxicity study

Table 1 shows the body weights at the start and end of the study and haematological findings in each group in wk 13. No animals in any group died during the experimental period. Weight gains were less in all experimental groups than in the control group from the first week of the experiment, and in wk 13 the weight gains of the 1.25, 2.5, 5, 10 and 20% groups were 89, 94, 84, 76 and 76%, respectively, of that of controls for males and 96, 98, 80, 84 and 92% for females. Except in the male 2.5% group and female 1.25, 2.5 and 20% groups, the differences in body weights from the controls were significant in wk 13. No adverse effects of caramel on haematological findings were observed either during or at the end of the study. At autopsy, there were no pronounced macro-

Table 1. Body weights and haematological findings in Fischer 344 rats given ammonia-process caramel in their drinking water for 13 wk

Sex and dose (%)	Body weight		RBC (10^4/mm^3)	Total WBC (10^2/mm^3)	Differential WBC count (%)			
	Initial	Final			N	L	M	E
Male								
0	134.79 ± 6.43	307.41 ± 14.64	841.10 ± 45.83	70.60 ± 8.90	11.90 ± 4.81	83.00 ± 5.40	4.50 ± 1.63	0.60 ± 0.66
1.25	136.00 ± 6.46	289.12 ± 14.09*	844.20 ± 64.59	72.40 ± 7.13				
2.5	135.74 ± 5.92	297.76 ± 14.14	878.40 ± 80.86	69.90 ± 15.09				
5	134.23 ± 6.38	279.73 ± 16.56**	862.70 ± 55.56	66.60 ± 9.43				
10	133.50 ± 6.50	265.11 ± 12.33**	892.60 ± 63.41	77.90 ± 4.01				
20	135.49 ± 5.57	265.83 ± 13.30**	874.10 ± 50.56	79.30 ± 6.25*	12.10 ± 3.36	85.50 ± 3.72	2.10 ± 1.04	0.30 ± 0.46
Female								
0	109.33 ± 4.03	173.29 ± 7.55	738.30 ± 88.64	55.20 ± 7.97	15.30 ± 7.71	81.40 ± 8.33	2.50 ± 1.12	0.80 ± 0.75
1.25	108.82 ± 3.78	170.08 ± 4.65	777.30 ± 65.76	59.90 ± 7.74				
2.5	109.27 ± 4.15	172.11 ± 11.14	771.80 ± 57.12	63.10 ± 6.83				
5	108.91 ± 4.10	160.06 ± 7.93**	782.80 ± 51.31	64.50 ± 5.50*				
10	109.39 ± 4.26	163.17 ± 8.37*	796.90 ± 55.28	61.60 ± 8.40				
20	109.53 ± 3.57	168.51 ± 4.99	781.00 ± 29.53	65.20 ± 7.01*	10.00 ± 4.65	87.20 ± 4.09	2.10 ± 1.14	0.70 ± 0.64

N = Neutrophils L = Lymphocytes M = Monocytes E = Eosinophils

Values are means ± 1 SD for groups of ten animals and those marked with asterisks differ significantly (chi-square test) from the corresponding control values (*$P < 0.05$; **$P < 0.01$).

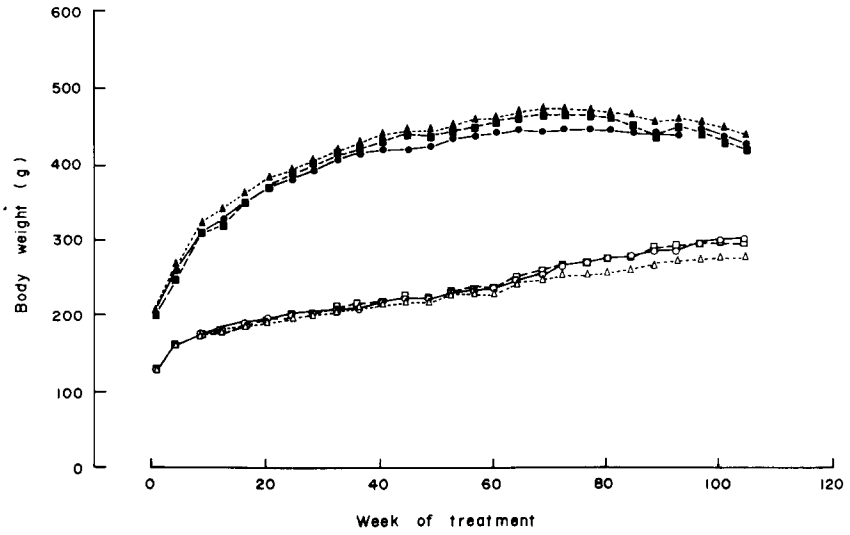

Fig. 1. Growth curves of male (closed symbols) and female (open symbols) F344 rats given ammonia-process caramel at 0 (●, ○) 1 (▲, △) or 4% (■, □) in the drinking water for 104 wk.

scopic changes in any animals, although a few rats in treated groups were very emaciated. No histological changes related to caramel administration were found in any experimental groups.

It was concluded that the maximum tolerated dose of caramel in the drinking water was between 2.5 and 5% for F344 rats, because the depression of weight gains was more than 10% compared with the controls in groups of both sexes given 5% caramel or more (except in females given 20% caramel). Therefore, 1 and 4% in drinking water were thought to be appropriate dose levels for the carcinogenicity study.

Carcinogenicity study

Growth curve and mortality. Figure 1 shows the growth curves of animals in each group. No significant differences were seen between the groups in either sex. Table 2 shows the cumulative mortality of tumour-bearing rats in each group. Tumours were found slightly earlier in the 1% group in males and

Table 2. Cumulative mortalities of tumour-bearing rats given ammonia-process caramel in their drinking water for 104 wk

Wk no.	Males			Females		
Caramel dose...	0%	1%	4%	0%	1%	4%
52				1		
56				1		
60		1		1		
64		1		2	1	1
68		1		2	1	1
72		2		2	1	3
76	1	4		2	1	5
80	1	4		2	2	6
84	1	5		4	4	9
88	3	6	3	5	5	11
92	4	9	4	5	5	14*
96	5	11	6	8	6	17
100	6	16*	7	9	8	18
104	7	16	14	10	10	21*
108	13	22	14	14	15	21
112	18	26	19	18	17	22
113	49	50	50	38	36	38

*Values marked with asterisks differ significantly (chi-square test) from the corresponding control values ($P < 0.05$).

Table 3. Caramel intakes, tumour incidences and mean survival times

Dose (%)	Sex	Effective no. of rats*	Total caramel intake in 2 yr (g/rat)	No. of rats with tumours (%)	Mean survival time ± SD and range (wk)
0	M	49		49 (100)	108 ± 8.3 (74–113)
	F	50		38 (76)	105 ± 14.6 (52–113)
1	M	50	153.3	50 (100)	103 ± 13.4 (58–113)
	F	50	116.8	36 (72)	106 ± 12.7 (63–113)
4	M	50	627.8	50 (100)	108 ± 8.1 (85–113)
	F	50	518.3	38 (76)	101 ± 15.2 (64–113)

*All groups initially comprised 50 rats and all those that survived beyond wk 52 were included in the effective numbers except one rat in which autolysis was too great.

the 4% group in females than in other groups. The cumulative mortalities of rats with and without tumours at the end of the administration period in the control, 1 and 4% groups were respectively, 16, 32 and 28% in males and 24, 24 and 42% in females.

Caramel intake, tumour incidence and mean survival time. The caramel intakes, incidences of all tumours, including benign and malignant tumours and mean survival times in each group are shown in Table 3. The first tumour-bearing rat was autopsied at wk 52 in the female control group. All rats surviving beyond this week were included in effective numbers except one male rat in the control group that died at wk 96, in which autolysis was strongly advanced. Throughout the administration period, daily water consumption in experimental groups was slightly more than that in the control group in both sexes, but was almost constant in each group. From these consumption data, the total caramel intakes in 2 yr were calculated to be as shown in Table 3. The incidence of tumours for all groups combined was 100% in males and 72–76% in females. There were no significant differences between the control and treated groups in total tumour incidences or mean survival times.

Organ distribution of tumours and histological findings in tumours. Table 4 summarizes the distribution and the histology of tumours found in this study. As shown in the Table, in males in all groups, tumours of the testis were the most frequent, followed by those of the mammary gland, haematopoietic organs, thyroid gland, lung, adrenal gland and pituitary gland. In females tumours of the uterus, mammary gland, haematopoietic organs and pituitary gland were frequent. Tumours were also detected in other organs and tissues in all groups, but at low incidences. In the experimental groups only the incidence of tumours of the pituitary gland in males showed a dose-related incidence and it was significantly higher in the 4% group than in the controls (chi-square test, $P < 0.05$).

Histological examination showed that all testicular tumours were benign interstitial-cell tumours which were the most frequent spontaneous tumours in F344 rats. Most uterine tumours were endometrial stromal polyps protruding into the lumen of the uterus with a stalk. A few adenomas, adenocarcinomas and endometrial sarcomas of the uterus were also observed and the incidences of malignant tumours were slightly, but not significantly, increased in the 4% group. The tumours observed in haematopoietic organs were so-called mononuclear-cell leukaemias characterized by marked splenomegaly and increase of atypical mononuclear cells resembling monocytes or large lymphocytes in the peripheral blood, except for two thymic leukaemias in the 4% male group. Most of the pituitary tumours were chromophobic adenomas, but some resembled eosinophilic adenomas. The mammary tumours differed histologically in males and females. In males the epithelial elements of fibroadenomas were strongly atrophic and only the mesenchymal elements proliferated like fibromas, whereas typical fibroadenomas were the most frequent in females. In the lung, adenomas originating from alveolar cells or terminal bronchioles were the most frequent. The thyroid tumours were predominantly C-cell adenomas originating from parafollicular cells and in a few cases C-cell carcinomas with metastases in remote organs were also found. The adrenal tumours were relatively small phaeochromocytomas, although there were a few malignant phaeochromocytomas with metastases in remote organs such as the lung.

Histologically other tumours were also similar to the spontaneous tumours reported by others (Altman & Goodman, 1979; Goodman, Ward, Squire *et al.* 1979). In addition to these tumours, many kinds of non-neoplastic lesions, such as myocardial fibrosis of the heart, bile-duct proliferation and fatty degeneration of the liver and chronic nephropathy of the kidney, were observed in all groups including the controls. No lesions specifically caused by caramel administration were detected. Biochemical and haematological examinations at the end of the study also showed no abnormal changes caused by caramel in any experimental group.

Age-related incidence of tumours. Table 5 shows the age-related incidence of tumours of the testis, uterus, mammary gland, haematopoietic organs and pituitary gland. These tumours increased rapidly after about 2 yr in all groups. Tumours of the uterus and haematopoietic organs in the 4% female group seemed to appear slightly earlier than those in the control group, although induction time of pituitary tumours in the 4% male group was similar to that in the other two groups.

In males in the satellite groups, no tumour was found in any rats of the control or 4% group killed at wk 26. In wk 52, interstitial-cell tumours were found in one of five rats in both the control and 4% group.

Table 4. Organ distribution and histological diagnosis of tumours observed in F344 rats given ammonia-process caramel in their drinking water for 104 wk

Organ/tissue affected and tumour type	No. of animals affected					
	Males			Females		
Caramel dose...	0%	1%	4%	0%	1%	4%
Genital system						
Testis: interstitial cell tumour	49	48	50	—	—	—
Uterus: endometrial stromal polyp	—	—	—	8	13	4
adenoma	—	—	—	1	0	1
adenocarcinoma	—	—	—	0	1	3
endometrial sarcoma	—	—	—	1	1	4
Mammary gland: fibroma	7	7	5	0	0	0
fibroadenoma	5	4	1	7	7	7
adenoma	1	0	0	2	0	0
adenocarcinoma	1	0	0	0	2	1
Endocrine system						
Pituitary gland: adenoma	3	5	13*	7	10	6
Thyroid gland: C-cell adenoma	6	6	5	1	3	2
C-cell carcinoma	1	1 (1)	2	0	0	1
papillary adenocarcinoma	0	0	0	0	1	0
Adrenal gland: phaeochromocytoma	5	5	7	1	3	3
malignant phaeochromocytoma	1	0	1 (1)	0	0	0
Pancreas: insuloma	3	1	5	2	2	0
Integument, musculoskeletal system						
Skin: papilloma	0	0	0	1	0	0
squamous-cell carcinoma	0	0	0	1 (1)	0	0
Subcutis: fibroma	2	2	2	0	0	1
lipoma	1	0	0	0	0	0
malignant fibrous histiocytoma	0	2	2 (1)	0	0	0
Bone: osteosarcoma	1 (1)	0	0	0	0	0
Preputial/clitoral gland: adenoma	3	4	1	3	0	0
Haematopoietic system						
Haematopoietic organs: mononuclear cell leukaemia	8	11	8	8	6	11
thymic leukaemia	0	0	2	0	0	0
Spleen: haemangioendothelioma?	1	0	0	0	0	0
Digestive system						
Tongue: papilloma	0	0	1	0	0	0
Small intestine: haemangioma	0	0	1	0	0	0
adenoma	0	1	0	0	0	0
adenocarcinoma	0	1 (1)	0	0	0	0
Large intestine/rectum: adenoma	1	1	0	0	1	0
Liver: neoplastic nodule	3	1	0	1	1	0
hepatocellular carcinoma	0	0	1	0	0	0
Pancreas: acinar-cell adenoma	1	1	0	0	0	0
Respiratory system						
Nasal cavity: squamous-cell carcinoma	0	0	0	1 (1)	0	0
Lung: adenoma	7	3	5	2	0	1
adenocarcinoma	0	0	1 (1)	1	0	0
unclassified sarcoma	0	0	0	1	0	0
Urinary system						
Kidney: adenoma	1	0	0	0	0	0
liposarcoma	0	1	0	0	0	0
transitional-cell carcinoma	0	0	1 (1)	0	0	0
Nervous system						
Brain/spinal cord: astrocytoma	0	2	1	0	0	0
Special senses						
Ear duct (Zymbal gland): squamous-cell carcinoma	0	0	0	2	0	1
Abdominal cavity						
Peritoneum: mesothelioma	2	2 (1)	3 (1)	0	0	0
Omentum: angiosarcoma	0	0	0	0	0	1
Retroperitoneum: angiosarcoma	1	0	0	0	0	0
liposarcoma	0	0	0	0	1	0

The value marked with an asterisk differs significantly (chi-square test) from the corresponding control value (*$P < 0.05$). No. of rats in parentheses are animals with metastases to remote organs. All groups initially comprised 50 animals and all were examined except one in the male control group, which was too badly autolysed.

Table 5. Age-related incidence of tumours in F344 rats given ammonia-process caramel in their drinking water for 104 wk

Age (wk)	Caramel dose...	No. of animals with tumours of the: Testis or uterus 0%	Testis or uterus 1%	Testis or uterus 4%	Mammary gland 0%	Mammary gland 1%	Mammary gland 4%	Haematopoietic organs 0%	Haematopoietic organs 1%	Haematopoietic organs 4%	Pituitary gland 0%	Pituitary gland 1%	Pituitary gland 4%
					Males								
<52									1				
53–65		1	2			1			1				
66–78		2	5	4		1			1	1		1	1
79–91		4	7	4	1		4	2	3	4		1	4
92–104		42	34	42	13	9	2	6	5	5	3	3	8
>104		109	105	108	111	107	108	107	96	104	111	103	108
	Mean induction time...												
					Females								
<52		1	1	1							1		
53–65							1			3			
66–78		1		4		1		1	2	2		2	1
79–91			1	3		2	2	2	1	5	1	1	1
92–104		8	13	4	9	6	5	5	3	1	5	7	4
>104		105	108	96	112	106	104	104	98	89	101	105	106
	Mean induction time...												

and a pulmonary adenoma was also detected in one of five rats in the 4% group. In wk 78, interstitial-cell tumours were observed in all rats of the control and 4% groups. On the other hand, no tumour was detected in females of any group. No hyperplasia or pre-neoplastic change was observed in the pituitary gland in any group.

DISCUSSION

Caramel is widely used in Japan and throughout the world as a food colouring. Recent studies have shown that ammonia-process caramel is weakly mutagenic and that its total consumption in Japan is relatively great (Kawachi *et al.* 1980). It therefore seemed important to investigate its carcinogenicity.

Gaunt, Lloyd, Grasso *et al.* (1977) reported that rats showed dose-related lymphocytopenia when given ammonia-process caramel in their diet (4, 8 or 16%) for 13 wk. Quite recently Spector & Huntoon (1982) reported that ammonia-process caramel contains a heat-stable competitive inhibitor of pyridoxal kinase. However, further studies demonstrated that lymphocytopenia in rats fed ammonia-process caramel was prevented with the addition of sufficient (8–12 ppm) pyridoxine to the diet (Sinkeldam, Roverts & Kuper, 1980). The basic diet used in this study contained sufficient pyridoxine (pyridoxine level in raw materials in the diet, 0.5–0.6 mg/100 g; pyridoxine level added to raw materials, 0.64 mg/100 g; total pyridoxine level in diet, 1.14–1.24 mg/100 g or 11–12 ppm), and there was no evidence of lymphocytopenia in rats given ammonia-process caramel in either the subchronic toxicity study or the carcinogenicity study. These findings are consistent with those of Sinkeldam *et al.* (1980) and Heidt & Rao (1981).

Evans, Butterworth, Gaunt & Grasso (1977) reported that there was no evidence of carcinogenic effect in Wistar-derived rats given 1, 3 or 6% caramel, product by the 'half open–half closed pan' ammonia process, in the diet for 2 yr, although body-weight gain was reduced at all dose levels in males and lymphocytopenia was observed in the high-dose males until wk 52.

In our study, many different types of tumours were observed in all groups including the controls and the organ distribution of these tumours and their histological characteristics were similar to those of the spontaneous tumours described by others (Altman & Goodman, 1979; Goodman *et al.* 1979). The incidences of tumours in the control group were also similar to those of historical controls in our previous studies on F344 rats, except for those of mammary tumours in females, pituitary tumours and leukaemias in both sexes, and thyroid and lung tumours in males (Maekawa, Ogiu, Onodera *et al.* 1982). The incidences of mammary tumours, pituitary tumours and leukaemias were slightly lower than observed previously, but were similar to those described by others (Goodman *et al.* 1979; Sher, Jensen & Bokelman, 1982; Tarone, Chu & Ward, 1981). On the contrary, the incidences of thyroid and lung tumours were slightly higher than those of our historical controls.

The only significant difference between control and experimental groups was in the incidence of pituitary tumours in males. The pituitary tumour is one of the commonest spontaneous tumours in F344 rats. Tarone *et al.* (1981) reported that considerable variation in control tumour rates was observed in experiments with F344 rats performed in six different laboratories, and pituitary tumours showed great variation in incidence. The incidence of pituitary tumours observed in the 4% male group in this study was within the limits of spontaneous tumour rates reported by others (Tarone *et al.* 1981; Sher *et al.* 1982), although the incidence was higher than that of the matched controls and also than that of the historical controls in our previous study. Moreover, most of the pituitary tumours detected in the 4% male group were very small microscopic tumours, and there was no significant difference in their induction times from those of controls. On the other hand, no abnormal changes were found in the pituitary gland in the satellite group given 4% caramel and there was no significant difference between hyperplastic or pre-neoplastic changes of the pituitary gland in control and experimental groups. From these findings, we conclude that the increase of pituitary tumours in the 4% male group is not attributable to caramel administration, but results from variation in the incidence of spontaneous tumours.

Thus it is concluded that ammonia-process caramel is not carcinogenic in F344 rats when administered continuously in the drinking water for 2 yr.

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