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Toxicology and Carcinogenicity Studies of Diuretics in F344 rats and B6C3F1 Mice 1. Hydrochlorothiazide

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Toxicology and carcinogenesis studies of hydrochlorothiazide, a benzothiadiazide diuretic, were conducted by administering diets containing the drug to both sexes of F344 rats and B6C3F1 mice in 15-day, 13-week and 2-year studies. No rats died during the 15-day or 13-week studies at dietary concentrations of up to 50 000 ppm. Deaths of male mice in the top dose group in the 13-week study were likely to be related to chemical administration. In the prechronic studies, increased nephrosis and mineralization at the kidney corticomedullary junction were the primary toxic effects of hydrochlorothiazide observed in rats. In mice, chemical-related effects included nephrosis and calculi, inflammation and epithelial hyperplasia in the urinary bladder. In 2-year studies using dietary concentrations of 0, 250, 500 and 2000 ppm in rats and 0, 2500 and 5000 ppm in mice, survival of dosed and control groups of rats and mice was similar, as were body weights of mice. Dosed groups of male and female rats were uniformly lighter than controls (up to 25%) throughout the studies. Severe chronic renal disease with secondary parathyroid hyperplasia and fibrous osteodystrophy of the bone were attributed to chemical administration in rats. No neoplasms in rats or female mice or non-neoplastic lesions in mice were associated with hydrochlorothiazide. In high-dose male mice, liver neoplasms were increased but were not considered to be related to hydrochlorothiazide administration because of an unusually low incidence in the control group relative to historical controls.

INTRODUCTION

Hydrochlorothiazide is one of several benzothiadiazides used primarily as a diuretic. It was synthesized during efforts to develop inhibitors of carbonic anhydrase, but its diuretic action is largely independent of this property.[1] Hydrochlorothiazide is used to lessen edema due to congestive heart failure, or chronic hepatic or renal disease, and is used in the treatment of hypertension.[2]

Hydrochlorothiazide is absorbed following oral administration, and binds to plasma proteins and red blood cells.[3,4] Peak plasma levels occur ca. 2 h after dosing.[5] The decline of plasma drug levels fits a two-compartment model with a terminal elimination phase of 9.5 h. The relatively long half-life provides a fairly constant, long duration of action (12–24 h).[6] Renal clearance is over 300 $m\ell\ min^{-1}$, indicating active secretion of the drug by the kidneys.[4] Secretion is via the organic anion transport system in the proximal tubules.[1] Urinary recovery of unchanged hydrochlorothiazide averages ca. 70% following oral administration and > 90% after i.v. administration. Hydrochlorothiazide apparently does not normally undergo metabolism. However, an unidentified non-renal excretion mechanism is active in patients with severe renal failure.[7]

Hydrochlorothiazide increases Na^+, $C\ell^-$, K^+, Mg^{2+} and water loss in the urine. The excretion of uric acid and Ca^{2+} are decreased relative to that of Na^+.[1] Thiazide diuretics can increase Na^+ excretion to 10–15% of the filtered load.[6] The primary site of action is the distal convoluted tubule; additional effects have been reported on resorption in the cortical and/or medullary collecting duct, and minor contributions to the diuretic effect may result from weak carbonic anhydrase inhibitory action in the proximal tubule.[8]

Other renal effects of hydrochlorothiazide include increased renin[9] and kallikrein[10] release. The latter action may be important in therapy for hypertension. Clinical experience has shown that hydrochlorothiazide has two major effects in the treatment of hypertension. One is an initial decrease in cardiac output owing to volume depletion from the diuresis, and the second is a fall in peripheral resistance occurring after a few weeks of therapy. The reasons for the prolonged response on peripheral resistance remain under investigation.[11,12]

Side effects of hydrochlorothiazide use include hypokalemia with resultant muscle cramps, cardiac arrhythmias, hyperglycemia and hyperlipidemia.[14] Electrolyte imbalances, in particular hypokalemia and hypomagnesemia, may be involved in sudden deaths in patients with pre-existing ventricular ectopy.[15] Results of the Multiple Risk Factor Intervention Trial, a ten-year

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multicenter study of factors involved in heart disease, indicated that high-dose hydrochlorothiazide (100 mg day^{-1}) therapy was associated with higher incidences of sudden death among patients with both high blood pressure and electrocardiographic abnormalities. The involvement of hypokalemia and hypomagnesemia in this observation remains controversial.[16–18]

Chronic hydrochlorothiazide therapy in humans increases Ca^{2+} retention and can lead to hypercalcemia.[19] A related finding is an association of hydrochlorothiazide treatment with hyperparathyroidism.[20] Thiazides may cause a primary hyperparathyroidism with reduced Ca^{2+} excretion and increased K^+ loss, owing in part to increased parathyroid secretion.[21,22] Glucose intolerance is a frequently encountered side effect of chronic thiazide therapy[24] and may be associated with hypokalemia.[23]

Immunological reactions to hydrochlorothiazide therapy include cases of severe allergic pneumonitis,[25] a photoallergic dermatitis resembling subacute cutaneous lupus erythematosus[26] and several types of hematological dyscrasias.[27] Neutropenia has been reported in several patients, with a pattern of onset that suggested a toxic depression of the bone marrow. Thrombocytopenia has been reported also with hydrochlorothiazide therapy and with other thiazides,[27–29] and appears to be immunologically mediated.

Hydrochlorothiazide is one of three diuretics under study by the National Toxicology Program (NTP). Hydrochlorothiazide was selected for study because of extensive human exposure to the drug, a lack of long-term animal studies and the potential for the drug to form *N*-nitroso derivatives under acidic conditions.[30] In genetic toxicity studies, hydrochlorothiazide was negative in the Salmonella assay and in the sex-linked recessive lethal assay in Drosophila. In cultured Chinese hamster ovary cells, hydrochlorothiazide induced sister chromatid exchange but not chromosomal aberrations.[31] This report summarizes results of 15-day, 13-week and 2-year feed studies with hydrochlorothiazide in F344 rats and B6C3F1 mice; complete details are available elsewhere.[31] An accompanying report[32] summarizes results of similar studies with furosemide. Studies of the toxicity and carcinogenicity of triamterene are ongoing.

EXPERIMENTAL

Chemicals

USP-grade hydrochlorothiazide, (6-chloro-3,4-dihydro-2*H*-1,2,4-benzothiadiazine-7-sulfonamide 1,1-dioxide) was obtained from Ciba Pharmaceutical Company, Summit, NJ. The identity of hydrochlorothiazide was confirmed by infrared, UV/VIS and nuclear resonance spectroscopy. The purity was determined to be > 98% by elemental analysis, thin layer chromatography, HPLC, titration of the sulfonamide groups and Karl Fischer water analysis (see Ref. 31). Hydrochlorothiazide was stable when mixed in feed and stored in the dark at 5°C. However, losses of up to 10% after 7 days were observed under animal room conditions, thus feeders were changed twice per week. Sixty-five of 67 formulated diets analyzed during the studies were within ± 10% of the target concentrations.

Experimental design

Four- to eight-week-old male and female F344 rats and B6C3F1 mice were obtained from Charles River Breeding Laboratories (Portage, MI) for all studies. Animals were housed five per polycarbonate cage with heat-treated hardwood chip bedding. Feed (Rodent Chow 5001, Ralston Purina Co., St. Louis, MO for 15-day studies; NIH-07, Zeigler Bros., Gardeners, PA for 13-week and 2-year studies) and water were available *ad libitum*. After 2- to 3-week acclimation periods, animals were distributed to weight classes and assigned to cages according to a table of random numbers. Numbers of animals per dose group and hydrochlorothiazide concentrations in the feed are given in Table 1. Body weights and clinical signs were recorded throughout the studies. At study termination, animals were sacrificed with carbon dioxide. Necropsies were performed on all animals and tissues were examined for gross lesions. Approximately 40 tissues per animal were preserved in 10% neutral buffered formalin, trimmed, embedded in paraffin, sectioned, stained with hematoxylin and eosin and examined microscopically for non-neoplastic and neoplastic lesions. Histopathological examination of tissues of female rats and of mice of each sex was performed according to the 'inverse pyramid' design.[34,35] Other details of pathology procedures and review processes have been reported elsewhere.[31,33]

Blood was collected from the heart of rats at a 52-week interim sacrifice in the 2-year studies and examined for differential blood count and erythrocyte morphology by standard methods. Plasma for clotting studies was frozen in dry ice–methanol prior to analysis for prothrombin time, activated partial thromboplastin time and fibrinogen content (see Ref. 31).

Statistical analyses

Differences in survival were analyzed by life table methods.[36] For analyses of tumor incidence data, three procedures were used to assess dose–response trends and to test for pairwise differences between dosed groups and controls:

(i) life table analyses (appropriate for fatal tumors);
(ii) the incidental tumor test (appropriate for tumors observed in animals dying from an unrelated cause);
(iii) the logistic regression test (also appropriate for incidental tumors, but does not require selection of time intervals for comparisons).

For further discussion of these statistical methods, see Haseman[37] and NTP.[31]

RESULTS

Prechronic studies

No rats or mice died during the 15-day studies. Body weight gains were variable and generally less at higher doses. No compound-related clinical signs or evidence of diuresis were observed. In rats, slight to moderate thymic hemorrhage was noted in 1/5–3/5 animals in each of the top three dose groups. Calculi were found

Table 1. Dietary concentrations used in hydrochlorothiazide studies.

15-Day studies (n = 5 animals of each sex per group)
Rats and mice—0, 3125, 6250, 12 500, 25 000 or 50 000 ppm

13-week studies (n = 10 of each sex per group)
Rats—0, 3125, 6250, 12 500, 25 000 or 50 000 ppm (1st study);
0, 250, 500, 1000, 2000 or 4000 ppm (2nd study)
Mice—0, 3125, 6250, 12 500, 25 000 or 50 000 ppm

2-Year studies (n = 50 per sex and dose for mice, 60 for rats with 10 sacrificed at 1 year)
Rats—0, 250, 500 or 2000 ppm
Mice—0, 2500 or 5000 ppm

in the urinary bladders of 1/5 or 2/5 mice in each of the top two dose groups.

In 13-week studies, no deaths occurred in rats, but 7/10 male and 1/10 female mice that received 50 000 ppm, 2/10 males at 25 000 ppm, 3/10 females at 12 500 ppm and 1/10 males and 3/10 female mice that received 3125 ppm died before the end of the studies. Microscopic evidence of pneumonia was found in several mice at the end of the study; autolysis and cannibalism prevented evaluation of several mice that died. Body weights were 11% lower in high-dose male mice and 9–24% lower in the top three dose groups of female mice. Compound-related effects in mice included increased incidences of nephrosis (kidney tubular cell degeneration and necrosis), calculi in the urinary bladder and inflammation and/or epithelial hyperplasia of the urinary bladder (Table 2). The calculi found in one bladder were analyzed and found to consist primarily of hydrochlorothiazide. Based on the incidence and severity of kidney and urinary bladder lesions, dietary concentrations of hydrochlorothiazide selected for mice for the 2-year studies were 2500 and 5000 ppm. Mortality information was not considered in dose selection because of uncertainty over the possible contribution of pneumonia to the deaths observed at lower doses.

The final mean body weights of rats were 7–16% lower than controls in all dose groups in the 13-week studies, and none of the clinical signs appeared to be chemically related. Mineralization of the kidney was observed in all dosed rats; this was most prominent at the corticomedullary junction and the severity appeared to be related to dose. Nephrosis was observed in 1/10 males and 5/10 females at the top dose. White crystals were found ocasionally on gross examination of the stomachs or urinary bladders of dosed rats. These crystals appeared to be physically similar to those analyzed from mice and were found to be primarily hydrochlorothiazide.

A second 13-week study was performed on rats using lower doses ranging from 250 to 4000 ppm hydrochlorothiazide in feed, in an attempt to determine the concentration at which there was no mineralization of the kidney. All rats survived to the end of the second 13-week studies and no clinical signs were chemically related. However, mineralization of the kidney again was observed at all doses. The severity was minimal to mild at the lowest dose. Uncertainty over the potential for this lesion to enhance the severity of nephropathy commonly seen in aging F344 rats led to the selection of three doses (250, 500 and 2000 ppm) for the 2-year study in rats.

Two-year studies

Rats. Ten male and female rats per dose group were sacrificed at 52 weeks. One of the 10 designated high-dose females died prior to 52 weeks with hemorrhage in the stomach, duodenum and pleural cavity. Nephropathy occurred in the 1-year animals, with increased severity in dosed rats; focal mineralization of the kidney was increased in mid- and high-dose male rats and in dosed female rats. In addition to the female rat that died in the 1-year study group, 16 dosed females in the 2-year study groups died prior to week 52. Of these 16 rats, 11 had internal hemorrhage. Because of this, a complete blood count and blood clotting tests were performed on the 52-week sacrifice animals. No significant compound-related hematological effects were observed, and measures of prothrombin times

Table 2. Number of mice with selected lesions of the urinary bladder and kidney in the 13-week hydrochlorothiazide studies.

	Males					Females				
Site/lesion	0	6250	12 500	25 000	50 000	0	6250	12 000	25 000	50 000
Urinary bladder										
Calculi[a]	0	0	3	2	7	0	0	4	9	6
Inflammation or epithelial hyperplasia	0	0	3	2/8	2/3	0	2/9	7/8	10	6/9
Kidney										
Nephrosis	0	0	2	2/8	3/3	0	0	5/8	9	9/9

[a] Calculi were observed grossly; other diagnoses reflect microcopic examination; n = 10 unless indicated otherwise.

and fibrinogen content appeared to be similar to control values in dosed male and female rats. Activated partial thromboplastin times were variable and appeared prolonged for certain dosed male rats and were unchanged for female rats (data not shown). However, no statistically significant changes in activated partial thromboplastin times were noted.

Mean body weights of dosed groups were similar and were 8–25% lower than controls throughout most of the 2-year studies (Fig. 1). Average feed consumption by dosed groups of rats was 88–94% of that by controls and was not related to dose. The estimated amount of hydrochlorothiazide consumed per day, averaged over the 2-year studies, was 10, 21 or 82 mg $kg^{-1}day^{-1}$ for low-, mid- or high-dose male rats and 12, 24 or 96 mg $kg^{-1}day^{-1}$ for low, mid- or high-dose female rats. No compound-related clinical signs were observed and there was no evidence of diuresis.

Estimates of the probabilities of survival for male and female rats in the hydrochlorothiazide studies are shown in the Kaplan and Meier curves in Fig. 2. No significant differences in survival were observed between any groups of either sex. Final survival was: males—control 18/50, low dose 16/50, mid-dose 9/50, high dose 11/50; females—control 31/50, low dose 26/50, mid-dose 30/50, high dose 27/50.

Chronic renal disease (nephropathy) was present in all groups of male and female rats (Table 3), but the severity was increased in dosed groups relative to controls. Nephropathy was characterized by degeneration and regeneration of tubular epithelium, tubular dilatation and atrophy, thickening of basement membranes, glomerulosclerosis and variable interstitial fibrosis and chronic inflammation. The incidence of cysts in the renal cortex and of hyperplasia of the transitional epithelium overlying the renal pelvis were also increased in dosed rats. Tubular cell adenomas were observed in 1/49 mid-dose and 1/50 high-dose female rats. The historical incidence of renal tubular cell neoplasms in untreated female F344 rats is 4/1928 (0.2%) in NTP studies.[31]

Lesions secondary to the increased renal disease were also noted in dosed rats (Table 3). Parathyroid hyperplasia, fibrous osteodystrophy and mineralization of multiple organs all occurred with increased incidences in dosed rats.

Fibroadenomas of the mammary gland in female rats occurred with a negative trend (Table 3), although the incidence in the control group is approximately twice that usually observed in NTP studies.[31] The lower incidences of these tumors in dosed female rats may have been influenced by the lower body weights attained by these animals compared to controls.[38]

Mice. Body weights and feed consumption of dosed and control males and females were similar. The average amounts of hydrochlorothiazide consumed per day were approximately 265 or 550 mg kg^{-1} for low- and high-dose male mice and 300 or 600 mg kg^{-1} for low- and high-dose female mice. No compound-related clinical signs were seen.

Estimates of the probability of survival for male and female mice are shown in Fig. 3. No significant differences were noted in survival of any group. Final survival was: males—controls 43/50, low dose 42/50, high dose 43/50; females—controls 38/50, low dose 40/50, high dose 35/50.

No non-neoplastic lesions in mice were attributed to hydrochlorothiazide administration. Hepatocellular

Table 3. Number of rats with selected lesions in the 2-year hydrochlorothiazide studies.

Site/lesion	Dose (ppm)							
	Males				Females			
	0	250	500	2000	0	250	500	2000
Number examined	50	49	50	50	50	50	49	50
Kidney								
Nephropathy	50	49	50	50	47	42	44	47
Cysts	2	19[a]	21[a]	18[a]	0	3	4	3
Mineralization of kidney or multiple organ	1	19[a]	27[a]	27[a]	10	40[a]	39[a]	40[a]
Epithelial hyperplasia of renal pelvis	6	21[a]	26[a]	23[a]	0	4	2	3
Tubular cell								
Hyperplasia	0	0	1	0	0	0	0	0
Adenoma	3	1	0	1	0	0	1	1
Parathyroid								
Hyperplasia	7	20[a]	30[a]	28/49[a]	0	12/47[a]	11[a]	10/49[a]
Bone								
Fibrous osteodystrophy	2	18[a]	23[a]	22[a]	0	9[a]	9[a]	5[a]
Mammary gland								
Fibroadenoma					30	12[a]	11[a]	5[a]

[a] $p < 0.05$ vs. control incidence.

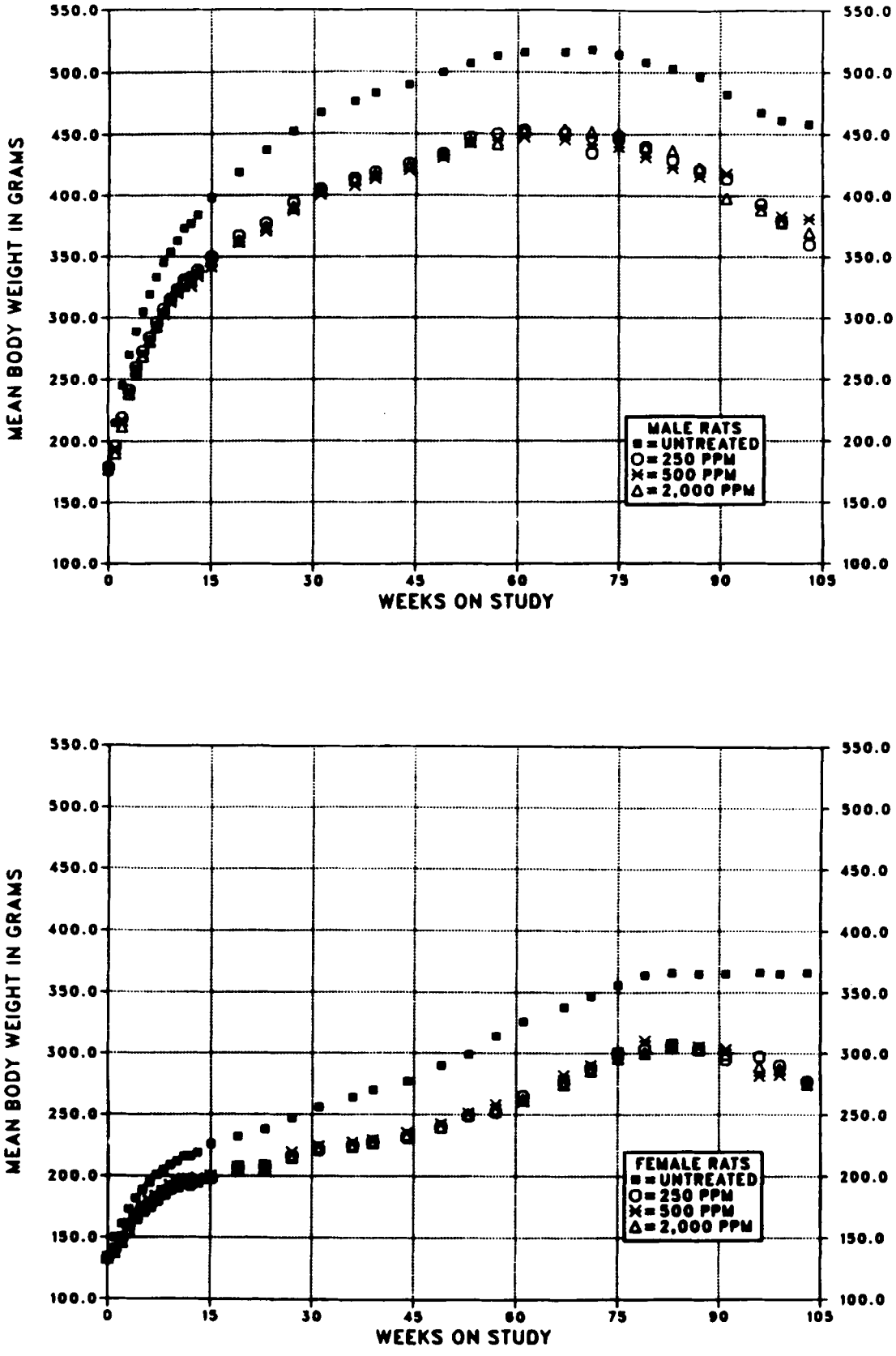

Figure 1. Weight gains of rats during the 2-year studies with hydrochlorothiazide.

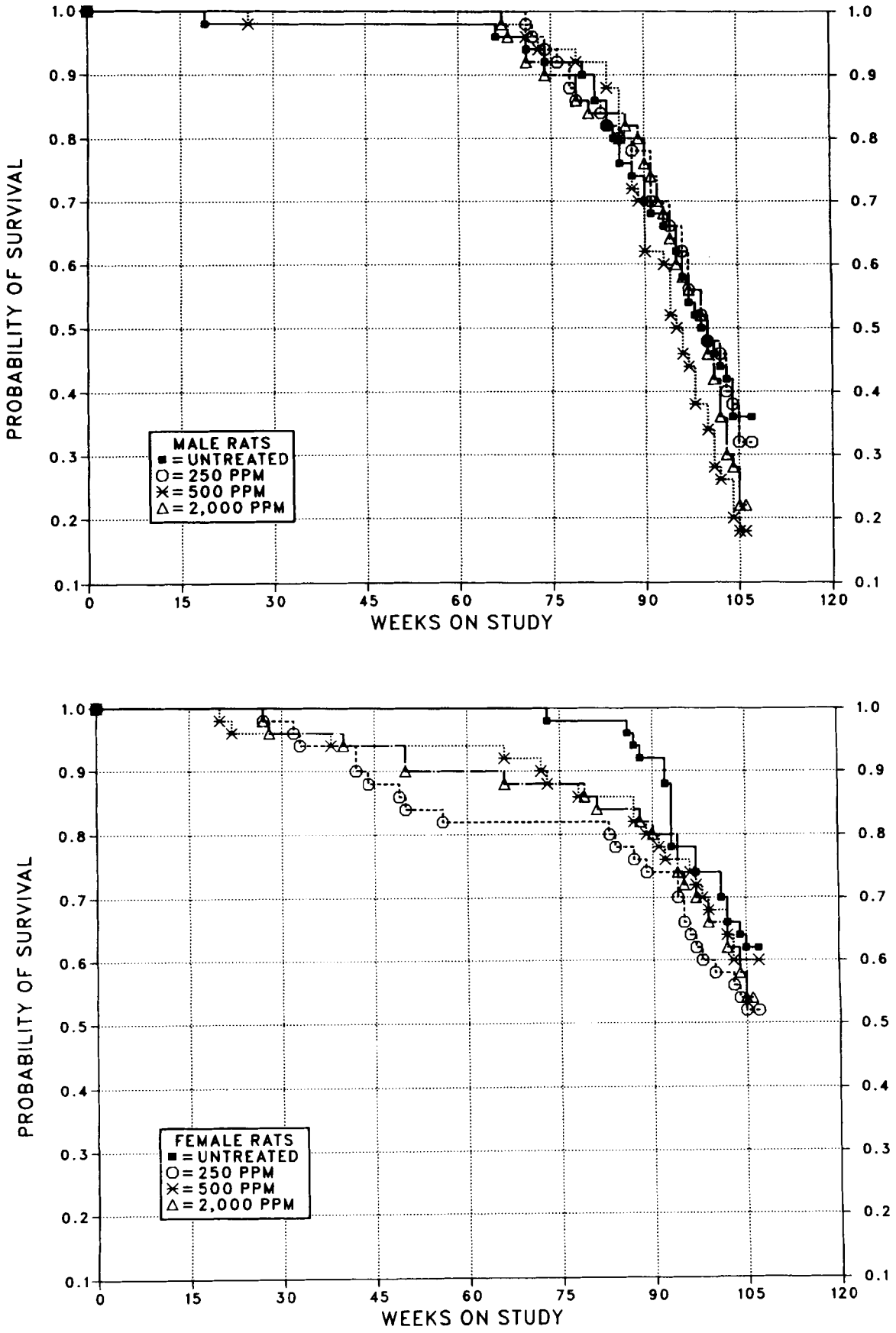

Figure 2. Kaplan–Meier survival curves for rats in the 2-year studies with hydrochlorothiazide.

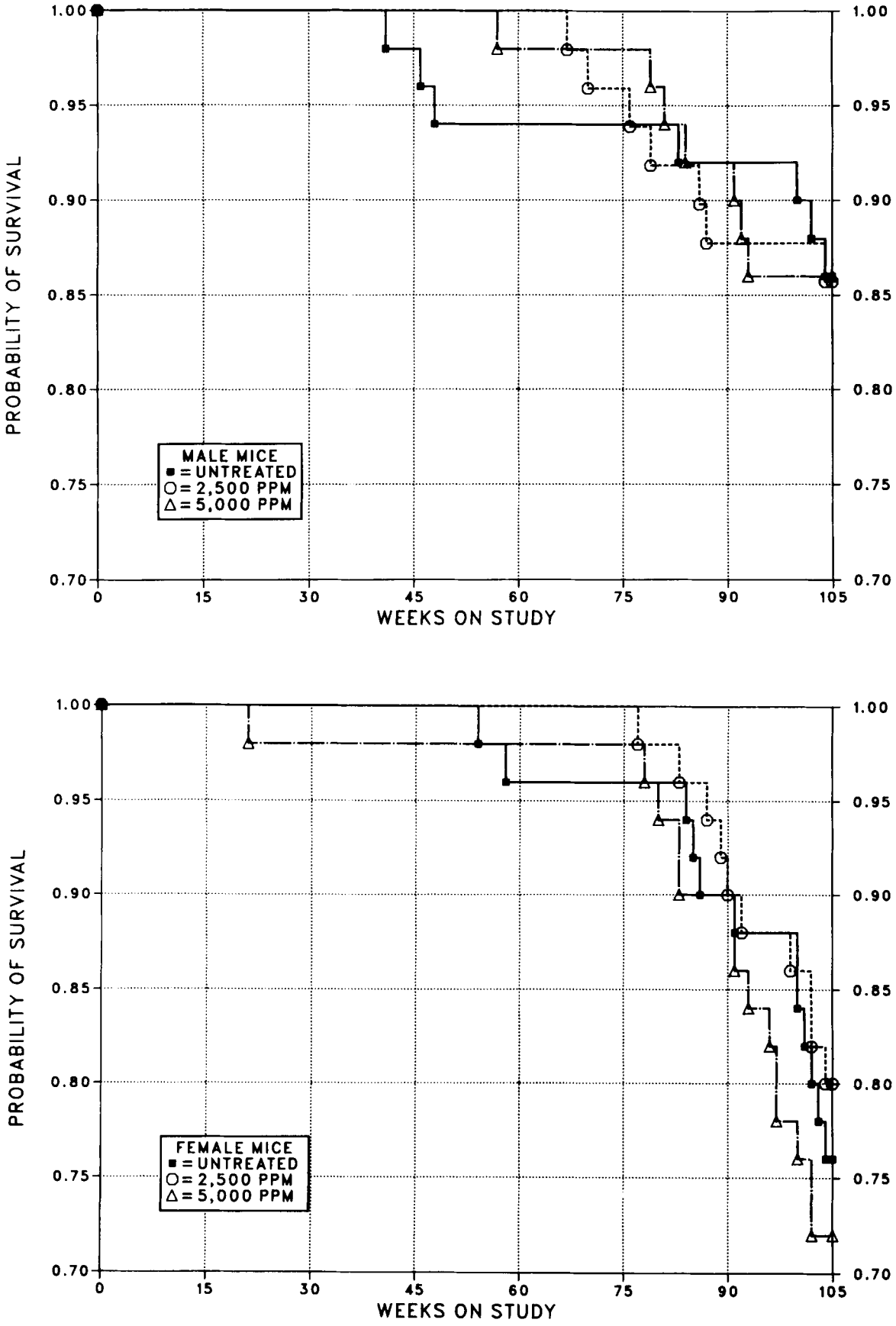

Figure 3. Kaplan–Meier survival curves for mice in the 2-year studies with hydrochlorothiazide.

adenomas (control 3/48, low dose 8/49, high dose 14/50) and adenomas and carcinomas combined (control 7/48, low dose 10/49, high dose 21/50) occurred with significant ($p < 0.01$) positive trends in male mice, and the incidences in the high-dose groups were greater ($p < 0.01$) than those in the controls. Hepatocellular carcinomas occurred with an incidence of 4/48, 4/49 and 9/50 in control, low- and high-dose male mice, respectively (n.s.). Metastasis to the lung was seen in two control and two high-dose males.

DISCUSSION

The kidney was the primary target of toxicity of hydrochlorothiazide in both rats and mice. In rats in the first 13-week study, mineralization was observed at the corticomedullary junction in all dose groups, and severe nephrosis was seen in the top dose group. Because an apparent no-effect level was not reached, the 13-week study was repeated using lower doses; however, mineralization was observed again in all groups down to the lowest dose of 250 ppm. These effects were seen also in the 2-year studies in rats with severe nephropathy found in dosed groups, along with an increased incidence across dose groups of mineralization in the kidney and multiple organs, and other lesions secondary to severe chronic renal disease (parathyroid hyperplasia and fibrous osteodystrophy). The lack of incremental dose effects on weight gain in the prechronic or chronic study, and the presence of mineralization in the kidneys at all doses, suggested that the dietary concentrations employed in these studies were above that needed to produce a maximal pharmacological effect in rats, and that the mineralization might be associated with increased calcium retention observed with the use of thiazide diuretics.[19] This could result from a primary hyperparathyroidism, as suggested by Pickleman *et al.*,[21] but parathyroid glands were not obviously enlarged in the prechronic studies. Clear signs of diuresis were not apparent during any of the studies. This could be due to the relatively mild diuretic effect of hydrochlorothiazide and/or to the development of adaptive or compensatory mechanisms to this effect.

In 13-week studies in mice, increased nephrosis and inflammation and/or epithelial hyperplasia and calculi in the bladders were seen in males and females at doses of 6250 ppm or higher. The calculi were similar to those noted in mice in the 15-day studies and were seen also in the bladder of one rat in the 25 000 ppm group in the 13-week studies. The calculi were determined to contain hydrochlorothiazide. It is not clear why they developed in some animals and not others, or why they were not confined to the high-dose groups. White crystals, possibly hydrochlorothiazide, were noted in the stomachs of some rats dosed at hydrochlorothiazide concentrations as low as 6250 ppm.

During the first year of the 2-year rat study, many of the dosed females dying early had evidence of internal hemorrhage. Thrombocytopenia has been reported as an idiosyncratic response to thiazide therapy in humans,[28] but platelet levels in dosed rats were not different from controls in the 52-week interim sacrifice animals. Plasma concentrations of prothrombin and fibrinogen were also unchanged from controls, and activated partial thromboplastin times were highly variable but were not significantly longer for dosed rats. Thus, the mechanism for this apparently sex-related effect remains to be resolved.

Survival of all groups of male rats was lower than that usually observed in 2-year studies;[39] however, survival was good until about week 93 of the study, and the steep decline in survival thereafter may be due in part to an aggressive moribund sacrifice practice in effect during these studies.

The results in rats are quite similar to those reported,[40] in which hydrochlorothiazide was given (with or without sodium nitrite) to groups of 24 male and 24 female F344 rats at a dietary concentration of 1000 ppm for 104 weeks. In this earlier study, hydrochlorothiazide administration was associated with an increase in the severity of nephropathy and with a spectrum of lesions secondary to the kidney effects, parathyroid hyperplasia, osteitis fibrosa of the bone and calcification of the aorta and other arteries and soft tissues. Two parathyroid gland adenomas were found in the 24 dosed male rats. Several tubular cell tumors of the kidney were observed in the dosed groups, and although none were seen in controls, the incidences were not statistically different from controls. This is in concordance with the current study observations of a low incidence of renal tubular cell tumors in dosed female rats and none in the controls. However, the incidence of tubular cell tumors of the kidney in the present study was higher in control male rats than in the treated animals.

Nephropathy was also increaed in male rats and in male and female mice in 2-year studies with furosemide.[32] Tubular cell tumors of the kidney were marginally increased in dosed male rats given furosemide.

Hepatocellular neoplasms were increased in male mice over controls in the 2-year studies. However, the incidence of hepatocellular tumors in the control male mice (15%) is the lowest incidence observed in contemporary studies in the NTP historical database,[32] and the total incidence of tumors seen in dosed male mice in this study (31/99, 31%) is similar to the average incidence in controls in all NTP studies (30%). In addition, the incidence in the high-dose male mice (42%) is within the historical range for hepatocellular tumors in male mice.[31] Thus, the apparent increase in these tumors cannot be attributed clearly to hydrochlorothiazide administration. Kidney lesions were not increased in dosed mice in the 2-year studies. The top doses used in the 2-year studies were less than half those that resulted in kidney effects in the 13-week studies.

In conclusion, the primary toxic effect of prolonged hydrochlorothiazide administration to rats was to increase the severity of chronic renal disease and secondary lesions. No non-neoplastic effects in mice or neoplasms in rats or mice could be attributed clearly to hydrochorthiazide.

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