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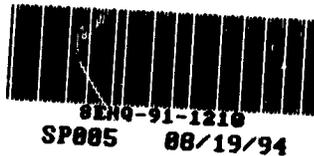


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**ANALYSIS OF STUDIES RELATED TO
TUMORIGENICITY INDUCED BY HYDROQUINONE**

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ABSTRACT

Hydroquinone (HQ) produced renal adenomas in male F344 rats, and these tumors appeared to arise from areas of spontaneous progressive nephropathy; the nephropathy itself was been found to be enhanced by HQ. Other neoplasms were not confirmed to be causally related to HQ among the reported bioassays. In the male F344 rat, HQ administered alone was not DNA reactive. HQ produced enhanced proliferation of renal tubular epithelium, presumably through toxicity involving glutathione conjugate formation. In the kidney, bone marrow and other tissues, HQ may induce toxicity by redox cycling and lipid peroxidation. In bone marrow, HQ may produce microtubulin dysfunction, which is a plausible explanation for positive cytogenetic tests, the only consistently positive genotoxicity effect reported for HQ. Although HQ is a metabolic product of benzene, several lines of evidence suggest that the effects of HQ exposure are significantly different from those of benzene. Based upon the plausible mechanisms by which HQ may produce kidney tumors in male rats, occupational exposure levels of HQ are not predicted to be a cancer risk for humans.

INTRODUCTION

Hydroquinone (HQ) (Figure 1) is a synthetic reagent used as a reducing agent in photographic developing, an antioxidant in the manufacture of rubber, a polymerization inhibitor for vinyl monomers, a chemical intermediate, and a depigmenting agent for skin blemishes. It also occurs naturally in complex mixtures such as cigarette smoke (Wynder and Hoffmann, 1967) and in plant-derived foods such as coffee, wheat-based products and red wine (Deisinger et al., 1994). The oxidation of HQ results in benzoquinone (BQ) via a semiquinone intermediate as shown in Figure 1.

In 1989 and 1991, two chronic bioassays (NTP, 1989; Shibata et al., 1991) reported induction of kidney adenomas in male F344 rats by HQ. Additionally, there were findings of mononuclear cell leukemia in female F344 rats, and liver adenomas in B6C3F₁ mice, which were not consistent between the two studies.

Two divergent but interrelated areas of research have developed regarding the effects of HQ. One area of investigation has been directed at the mechanism of formation of renal tubular cell adenomas in male F344 rats. These studies have attempted to determine whether HQ produces genetic alterations leading to neoplastic transformation, or produces tumors by HQ-mediated cell damage and proliferation in the kidney. In other research, HQ has been identified as a metabolite of benzene (Cox, 1991), and mechanistic studies have addressed whether there is a role for HQ in benzene-induced human leukemia. This review summarizes the bioassay data and analyzes information related to possible mechanisms for the tumorigenicity of HQ.

BIOASSAYS FOR TUMORIGENICITY

Early chronic studies of HQ (Carlson and Brewer, 1953) did not reveal any tumors in male or female Sprague-Dawley rats fed up to 10,000 ppm (approximately 500 mg/kg/day) for 103 weeks beginning at 23 to 24 days of age. The methods used and the descriptions of findings were not detailed except to note that histopathological findings were negative.

National Toxicology Program (NTP, 1989) Study Using Rats

The NTP (1989) reported results of administration of 0, 25, or 50 mg/kg/day HQ to male and female F344 rats by gavage 5 days per week, beginning at 7-9 weeks of age for up to 103 weeks. There was an interim sacrifice at 15 months. Doses were selected based on results of a 13-week study that revealed reductions in body weight gain, and forestomach and kidney lesions at doses higher than 50 mg/kg. These subchronic results will be described in the section on renal toxicity. In the chronic study, body weights of low and high dose males were significantly reduced.

Incidences of neoplasia showing differences between dosage groups are given in Table 1. Renal tubular adenomas in males were 0/55 (0%), 4/55 (7%), and 8/55 (15%) in the 0, 25, and 50 mg/kg/day groups, respectively. These tumors were characterized as small, discrete masses of epithelial cells arranged in solid clusters or nests. Location of lesions within the tubules was not specified in the NTP report. There were poorly defined tubular formations in some tumors. No kidney adenomas were reported in females. Only 2/55 (4%) of high dose males were reported to have renal tubular cell hyperplasia, and none was observed in any other group.

In high dose males, there was an increase in incidence of "marked" versus "moderate" spontaneous progressive nephropathy, a common condition in aged male rats. The "marked" nephropathy was characterized by papillary hyperplasia of the transitional epithelium overlying the renal papillae, and cysts. "Moderate" changes included varied degrees of degeneration and regeneration of tubular epithelium, atrophy and dilatation of tubules, hyaline casts and signs of chronic inflammation. The 15-month interim sacrifice showed an increase in spontaneous progressive nephropathy in males but not in females.

Females had incidences of mononuclear cell leukemia with splenic involvement in 9/55 (16%), 15/55 (27%), and 22/55 (40%) in the 0, 25, and 50 mg/kg/day groups, respectively (Table 1). The historical control incidence of this leukemia was reported to be 25% ± 15%. There was a dose-related increase in the severity of leukemia in females as scored by multiple organ involvement. There was a very high incidence of splenic mononuclear leukemia in male rats at 50-56%, with liver involvement in 47-55%, but these incidences were not dose related.

Although more pheochromocytomas of the adrenal gland were found in HQ-exposed male rats, these marginally significant findings were not considered to be related to HQ administration. No HQ-related increase in liver tumors was found.

NTP (1989) Study Using Mice

The NTP (1989) also reported results of a bioassay in B6C3F₁ male and female mice. Beginning at 8-10 weeks of age, animals were gavaged with 0, 50 or 100 mg HQ/kg/day, 5 days/week, for up to 103 weeks. Doses were selected based on decreased survival rate and increased forestomach lesions above 100 mg/kg in the preliminary 13-week study. There was an interim sacrifice at 15 months. In the chronic study, body weights were depressed in male and female high-dose groups with females showing a greater effect. At the 15-month sacrifice, liver weights were increased in high-dose males and females, and kidney weights were increased in low and high-dose females. At final sacrifice, low and high-dose males and high-dose females had increased liver weights, but increased kidney weights were not found.

The incidences of hepatic lesions found in this bioassay are shown in Table 2. These data indicate an increased incidence of hepatic adenomas in low and high dose female mice. While male mice showed an increased number of hepatic adenomas in low and high-dose groups, decreases in hepatocellular carcinomas versus controls were also reported. Consequently, no significant dose-related increases in total (combined adenomas and carcinomas) hepatic tumors occurred. Foci were not appreciably increased except in high-dose males, but even this number was low.

Other findings in mice included an increase in thyroid hyperplasia in males and females, and an increase in thyroid adenomas in females, which did not achieve statistical significance and was within the historical control incidence range for the NTP. No increased incidence of lymphoma or leukemia was found in mice.

Shibata et al. (1991) Study Using Rats

Shibata et al. (1991) investigated HQ effects in F344 rats of both sexes. Rats were given 0.8% HQ in the diet beginning at 6 weeks of age for 104 weeks. Calculated consumption was 351 mg/kg/day for males and 368 mg/kg/day for females. Weights of HQ-dosed animals were depressed for both males and females. There were increased liver weights in males, and increased kidney weights for both sexes. Tubular hyperplasias in the kidneys were present with stratified epithelial cells filling the tubular lumina. Renal adenomas were present in 14/30 (47%) of exposed males but not in females or male controls. Spontaneous progressive nephropathy appeared to be increased in severity by HQ, especially in males. Whereas no control males were found to have moderate or severe nephropathy, 9/30 (30%) and 5/30 (17%) of the exposed males were reported to have moderate and severe lesions, respectively. Livers of male and female rats given HQ showed a statistically significant decrease in the number of altered foci.

In contrast to the NTP studies, this high dose of HQ did not increase the incidence of hematopoietic neoplasias (Shibata, personal communication). Incidences of leukemia or lymphoma in females was 5/30 (17%) in controls, and 4/30 (13%) in the HQ-exposed group (Table 1). For males, incidences were 6/30 (20%) in controls, and 8/30 (27%) in the HQ-exposed group.

Shibata et al. (1991) Study Using Mice

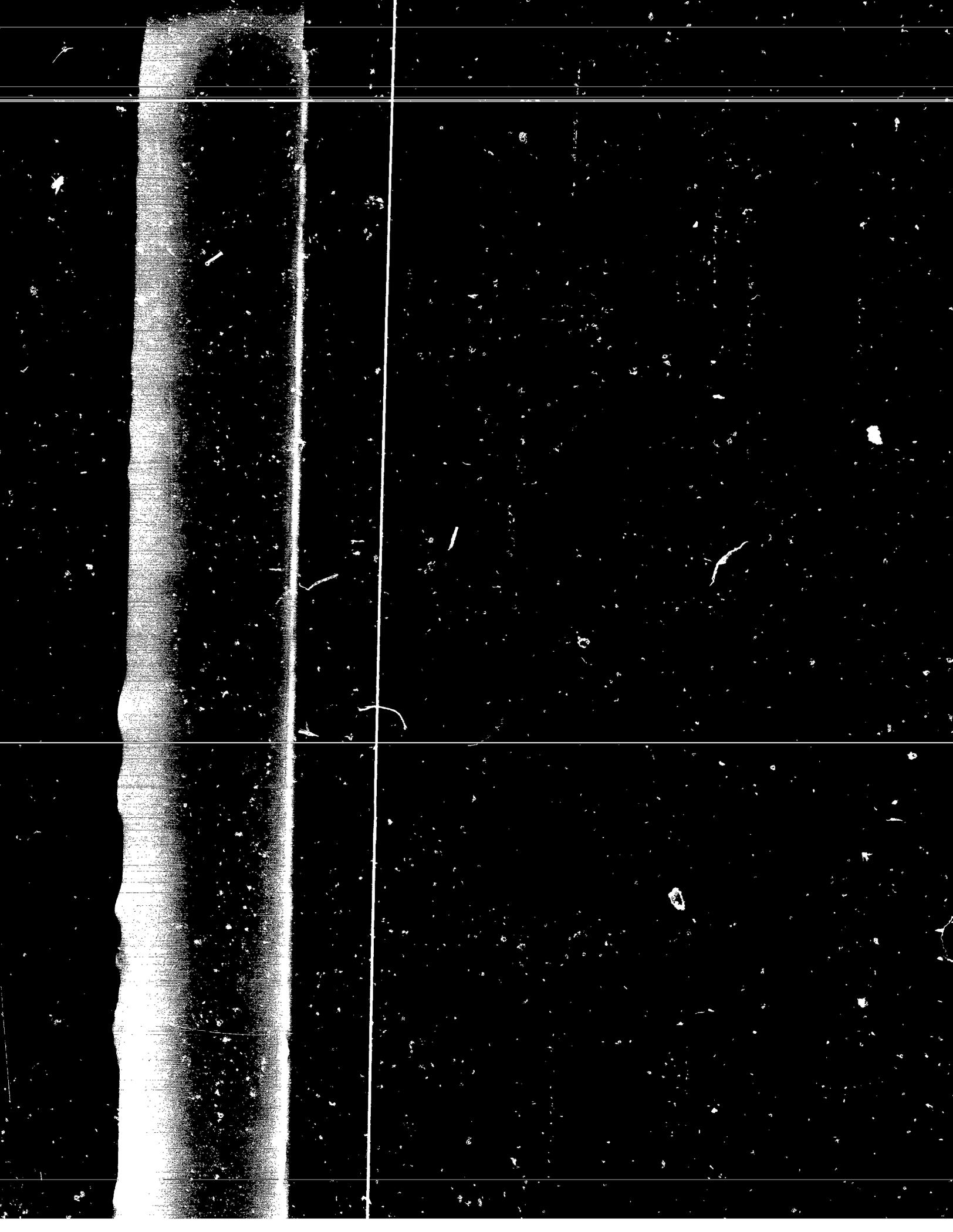
Shibata et al. (1991) investigated effects of HQ in B6C3F₁ mice of both sexes. Beginning at 6 weeks of age, mice were given 0.8% HQ in the diet for 96 weeks. Calculated HQ consumption was 1046 mg/kg/day for males and 1486 mg/kg/day for females. Males showed increases in liver hyperplasia, altered foci, and adenomas, but no change in incidence of carcinomas (Table 3). These effects were not found in females. The incidences

in males of renal tubular hyperplasia was 0/28 (0%) in controls, and 9/30 (30%) in the HQ-exposed group. Also, in HQ-dosed male mice, there was a 3/30 (10%) incidence of renal adenomas, but this latter finding did not reach statistical significance. The nonglandular stomach showed hyperplasia in both males and females given HQ.

ANALYSIS OF CANCER BIOASSAY DATA

A summary of animal bioassay findings from NTP (1989) and Shibata et al. (1991) along with our analysis of the tumorigenic potential of HQ are given in Table 4. The NTP concluded that there was some evidence of tumorigenic activity of HQ in male and female F344 rats and in female B6C3F₁ mice. The inference of tumorigenicity in rats was based on renal adenomas in males and on mononuclear leukemias in females. In female mice, the NTP conclusion was based on hepatocellular tumors. Shibata et al. found an increase in kidney adenomas in male rats, and liver adenomas in male mice, but not in female mice. In our analysis, evidence for tumorigenicity has been considered negative in this summary when completely inconsistent results were found between the two reported bioassays involving the same strain and sex of rat, and the positive result was within the range of reported historical controls. Evidence was considered equivocal if studies were contradictory within the same sex, species, and strain, tumors were common in controls, and either the highest dose study was negative or achieved marginal statistical significance only at the highest dose tested.

The finding of renal adenomas in F344 rats is consistent between the two bioassays (Table 1). In males, the higher incidence in the Shibata et al. (1991) study can be explained by use of a much larger daily dose and 7-day per week exposure. In neither study did females show any renal tumors. The finding of renal tubular cell hyperplasia and renal adenomas in mice is important because of the rarity of these pathological findings in this species. Incidence of renal adenomas in male mice administered 1046 mg/kg/day was 10%, whereas incidence of spontaneous renal tumors has been reported to be 0.18% in male B6C3F₁ mice (Hard, 1987). Again, these lesions were observed in the Shibata et al. (1991) study and not in the NTP (1989) study presumably because of the much higher exposure. The early study in Sprague-Dawley rats at 500 mg/kg/day was also negative (Carlson and



Brewer, 1953). Although there is no description of any pathological findings, the dose employed was the highest of any of the rat bioassays.

An independent review (provided by G. Hard of the American Health Foundation) of photographs of the renal pathology yielded tumor incidences that were largely consistent with the NTP results. Two of the adenomas in the low dose group were at the borderline of hyperplasia, but they could also be considered to be adenomas. One of the adenomas in the high dose group appeared to be a large atypical hyperplasia. One additional atypical hyperplasia was found in one of the high-dose rats who also had an adenoma. An attempt was made in this review to localize the neoplastic lesions to determine the segments of the renal tubule that were involved. Seven of the 14 lesions appeared at the outer stripe of the outer medulla (Zone 2 bordering on Zone 1). Of the remainder, two appeared to be deep in Zone 2, one was a large cystic adenoma spanning the cortex into the medulla, and four were in the cortex; it was not possible to determine whether these were in the medullary rays. Since tumors were found deep in Zone 2, it appeared that P₃ cells of the proximal tubule were involved; however, due to the cortical tumors, P₂ and perhaps P₁ segments were also considered to be targets.

The NTP found an HQ-related increase in the severity of spontaneous progressive nephropathy. Additionally, a further analysis of the NTP data has revealed a correlation between occurrence of adenomas and degree of nephropathy among individual animals within the bioassay (English et al., 1994b). An independent review of the slides has found that almost all of the adenomas and hyperplastic lesions appeared to arise within foci of the spontaneous progressive nephropathy (G. Hard, personal communication).

In contrast to the finding of renal adenomas, the conclusion that there is some evidence of increased mononuclear leukemia in female rats is problematic. The very high rates of mononuclear leukemia in control and dosed groups of male rats did not show an effect by HQ. Although the high dose group of females exhibited a statistically significant increased incidence of this neoplasm compared to controls, incidences in all dose groups were still within the range of historical controls. Furthermore, no increase in leukemia incidence was found (Shibata, personal communication) in either sex of F344 rats even though the dose in this study was 10 times that of the NTP study when calculated on a daily

basis (Table 1). No increased incidence of leukemia or lymphoma was found in either bioassay study of mice. Furthermore, Rao et al. (1990) have reported an unexplained increase in the incidence of mononuclear cell leukemia in F344 rat studies conducted at the NTP from 1971 through 1981, which has remained to the present. Consequently, the finding of mononuclear leukemia only in female rats from one study would not appear to constitute evidence of carcinogenicity, and we judge this finding to be unrelated to HQ exposure (Table 4).

Findings of hepatic adenomas in mice were not consistent between the two bioassays, and no increases in hepatocellular carcinomas were seen in either study (Tables 2 and 3). In the NTP study, there was no change in liver cancer incidence except for a decrease in high-dose males, which was not statistically significant. Consequently, in these HQ-exposed males, although there was a statistically significant increase in adenomas, the decrease in carcinomas resulted in no statistically significant HQ-related change in total number of liver tumors. In the study of Shibata et al. (1991), male mice demonstrated a statistically significant increase in adenomas but not in carcinomas. The increased incidence of adenomas was statistically significant at the $P < 0.05$, but not the $P < 0.01$ level of confidence. Since there is a high and variable historical incidence of hepatic adenomas in mice, this result provides only equivocal evidence of tumorigenicity. In females, the NTP reported an increased incidence of adenomas for both low and high HQ dose groups, and although these increases were not dose related, they were outside of the historical range. Shibata et al. (1991) found no increases in either adenomas or carcinomas for females at higher doses. Consequently, we judge the evidence to be equivocal for adenomas in female mice.

MECHANISTIC STUDIES OF RAT KIDNEY TUMORS

Identification of benign kidney tumors only in male rats, which may be associated with HQ-induced enhancement of age-related spontaneous progressive nephropathy or other renal toxicity mechanisms, has prompted several mechanistic studies. Only one reported study has directly investigated HQ reaction with DNA in the kidney *in vivo*. Other

mechanistic studies involving male rat kidney tumors have sought to investigate HQ-induced toxicity and enhancement of cell proliferation rates.

Absorption, Metabolism and Transport to the Kidney

HQ is biotransformed to water soluble sulfate, glucuronide and glutathione conjugates in the liver (DiVincenzo et al., 1984; Vamvakis and Anders, 1990; Monks et al., 1990). The sulfate and glucuronide conjugates are filtered by the kidney glomerulus and eliminated in the urine. The glutathione conjugate is also filtered but is selectively reabsorbed by the tubule where it may be nephrotoxic (Vamvakis and Anders, 1990; Monks et al., 1990).

When HQ was given in diet or by gavage, it was almost completely absorbed from the intestinal tract of male Sprague-Dawley rats, according to DiVincenzo et al. (1984). These investigators analyzed urinary metabolites after both a single gavage dose of 311 mg/kg ¹⁴C-HQ, and after a two-day regimen of 5.6% HQ in the diet. Virtually all (about 99%) of the ¹⁴C-HQ was recovered from urine, and small amounts were recovered from feces, breath and carcass. In the carcass evaluation, liver and kidney had the highest concentrations. In the urine evaluation, the glucuronide conjugate was found to comprise 63% of the radiolabel after gavage and 67% after the diet study. The sulfate conjugate was found to comprise 29% after gavage, and 25% after the diet study. The remainder was excreted as free HQ. Greenlee et al. (1981a) found that intravenous injection of ¹⁴C-HQ in male F344 rats resulted in HQ distribution to most organs, but principally to bone marrow, spleen and thymus. These studies suggest that route of administration may have an influence on the disposition of HQ. Radiolabeled HQ was found to be bound to liver and kidney macromolecules to a greater extent than bone marrow and blood when administered intraperitoneally to mice (Subrahmanyam et al., 1990).

English et al. (1988) studied the toxicokinetics of HQ in F344 rats. ¹⁴C-HQ was administered by gavage or dermally to male and female rats, 7 to 9 weeks of age. Single doses of 25 and 350 mg/kg were used in the oral study and doses of 25 and 150 mg/kg in the dermal study. Another group was dosed for 15 days with 25 mg/kg by gavage. Urinary metabolites were analyzed by HPLC. Radiolabel recovery in urine after 24 hours from the three oral dose groups was 87-94%, with little apparent difference between dose groups or

sexes. Approximately 1-3% was found in feces after 24 hours and less than 1% in tissues and carcasses after 48 hours, liver and kidneys having the greatest concentrations. In the dermal study, HQ was reported to be poorly absorbed. Analysis of urinary metabolites 24 hours after oral administration showed that the HQ glucuronide, sulfate, and mercapturate represented 45-53%, 19-33%, and 0-5%, respectively. Amounts of unconjugated HQ and BQ were 0-3% and less than 1%, respectively. In all of the recovery and metabolite studies, there were few discernable differences between sexes. An additional metabolite was found by Inoue et al. (1989a, 1989b) who showed that there was excretion of 1,2,4-benzenetriol in female Wistar rats and male Japanese white rabbits after intraperitoneal HQ administration. Thus, metabolism of HQ may also be influenced by route of administration.

Lunte and Kissinger (1983) investigated HQ-glutathione formation *in vitro* and found that it was enhanced by addition of mouse liver microsomes in the presence of NADPH. They also showed that spontaneous oxidation to BQ did not lead to significant glutathione conjugate production; however, addition of microsomes and NADPH produced a 100-fold increase in glutathione conjugation. Addition of cytosol alone produced a 10-fold increase. Cytochrome P450 involvement in this process, mediated by superoxide formation, had been postulated by Greenlee et al. (1981b), but in experiments by Lunte and Kissinger (1983), neither metyrapone, which inhibits cytochrome P450, nor superoxide dismutase prevented formation of the conjugate. In contrast, Hill et al. (1993) concluded, on the basis of inhibitor studies, that cytochrome P450 catalyzes a significant portion of hepatic HQ oxidation. Hepatic peroxidases also apparently contribute to HQ oxidation (Lunte and Kissinger, 1983; Hill et al., 1993). O'Brien (1988) hypothesized that conjugation to glutathione was peroxidase mediated without the utilization of oxygen.

Hill et al. (1993) found glutathione conjugates of HQ formed *in vivo* and *in vitro* in the presence of rat liver microsomes. In the *in vivo* studies, after prior treatment with acivicin to block γ -glutamyl transpeptidase (γ -GT) activity, 1.8 mmol/kg (200 mg/kg) HQ was administered to rats intraperitoneally, and bile and urine were analyzed by HPLC. They found mono-, two di-, and triglutathione conjugates of HQ in bile and the N-acetylcysteine conjugate in urine. Together, these conjugates amounted to 4% of the dose. The authors suggested that the quantity of HQ glutathione conjugates formed after *in vivo* parenteral

administration of HQ was sufficient to propose a role for these metabolites in HQ-induced nephrotoxicity and nephrotumorigenicity. These investigators also found that infusion of the trigluthathione conjugate into *in situ* perfused kidney resulted in 100% retention.

Study of HQ-DNA Adducts in the Male Rat Kidney

English et al. (1994a) did not find DNA adduct formation by HQ in F344 rat kidney using the P_1 -enhanced ^{32}P -postlabeling technique. Male and female rats were given 0, 2.5, 25, or 50 mg/kg/day HQ by oral gavage 5 days per week for six weeks. (The high dose regimen was sufficient to produce mild kidney toxicity in the males). No DNA adducts were detected in kidney nuclear fractions isolated from rats administered HQ, determined by absence of additional chromatographic spots compared to unexposed animals. Positive controls for HQ and BQ DNA adducts were prepared by reaction of HQ with cultured rat Zymbal glands, and BQ with unexposed rat kidney DNA. These *in vitro* reactions resulted in several new spots on the chromatograms, indicating that adducts had been formed under these conditions. The detection limit was reported to be one adduct per 10^9 to 10^{10} nucleotides, and these *in vitro* positive controls yielded 900-1700 adducts per 10^9 nucleotides.

Renal Toxicity, Cell Proliferation, and Tumor Promotion

The NTP (1989) reported results of a 13 week study of HQ in male and female F344 rats. Animals, 7-8 weeks old, were gavaged with 0, 25, 50, 100, or 200 mg/kg/day HQ 5 days per week and were killed 1 or 4 days following the final dose. Toxic nephropathy was found in 7/10 high dose males, 6/10 high dose females, and 1/10 females dosed at 100 mg/kg. The lesions were reported to consist of tubular cell degeneration and regeneration in the renal cortex and were said to be more severe in males than in females. No evidence of hyaline droplets in renal tubular epithelium was reported although sacrifice of the HQ dosed males did not take place until four days after cessation of HQ administration, at which point HQ-induced hyaline droplets may have disappeared. A similar study was performed in B6C3F₁ mice at doses of 0, 25, 50, 100, 200 and 400 mg/kg (NTP, 1989). The only finding reported, except for poor survival in the high dose group, was an increase in liver weight

that was more pronounced in males at some doses. No histopathological lesions were reported in the livers.

The ability of HQ to produce lipid peroxidation and liver damage was studied by administration of 100 or 200 mg/kg to male Sprague-Dawley rats. Glutathione depletion occurred within two hours in the liver, and increased malondialdehyde was found in the urine excreted for 18 hours afterwards (Ekstrom et al., 1988; Stenius et al., 1989). HQ administration for 7 weeks at 100 mg/kg/day enhanced production of enzyme-altered liver foci in N-nitrosodiethylamine-initiated male Sprague-Dawley rats (Stenius et al., 1989). In addition, it has been shown that in isolated hepatocytes, HQ causes cell death, preceded by glutathione depletion, decreased protein sulfhydryl groups, and lowered ATP levels (Nakagawa and Moldeus, 1992).

Nephrotoxicity of HQ has been compared between male and female F344 rats, Sprague-Dawley rats and B6C3F₁ mice (Boatman et al., 1992). Increases in several indicators of nephrotoxicity were noted in urine of both male and female F344 rats, 8 hours after the 400 mg/kg dose. These indicators included alanine aminopeptidase, N-acetylglucosaminidase, alkaline phosphatase, γ -GT and glucose. In contrast, these effects were not observed in Sprague-Dawley rats nor in mice of either sex.

The effects of HQ and other dihydrobenzenes on cell proliferation and tumor promotion were studied in male rats (Okazaki et al., 1993). In a short term experiment in F344 rats, 2% HQ was administered in the diet for 3, 7, or 14 days, and one hour prior to killing, newly synthesized DNA was labeled by the administration of bromodeoxyuridine (BrdU). No change in the labeling of cells in the outer renal cortex and a decrease in hyaline droplets were reported. No increase in thiobarbituric acid reactive substance values in kidney was found 3, 6, 12, 24, or 48 hours after a single dose of 300 mg/kg HQ, whereas KBrO₃ did increase the values. Wistar/Crj rats were administered the initiating agent N-ethyl-N-hydroxyethylnitrosamine at 0.1% in drinking water for 3 weeks. HQ or other dihydrobenzenes were given at 0.8% in the diet for 36 weeks. Although no significant difference in the number of rats with renal tumors was found due to high renal tumor incidence in the control, the numbers of microadenomas and renal cell tumors per rat were increased by HQ compared to controls.

A study of HQ effects on renal tubular cell proliferation has been reported by English et al. (1994b). In a six week study, which was designed to mimic the initial time period of the NTP bioassay study design, HQ was administered by gavage to male and female F344 rats at doses of 0, 2.5, 25, or 50 mg/kg/day, five days per week. Sprague-Dawley males were similarly dosed at 0 and 50 mg/kg/day to examine strain differences. Cell proliferation rates were measured after 1, 3, and 6 weeks by incorporation of BrdU, which was administered by osmotic pump for three days before sacrifice. Also, English et al. (1994b) reported on urinary parameters of tubular cell damage. These parameters were measured at 8 and 24 hours after HQ administration, and kidneys were examined histopathologically. After 6 weeks of 50 mg/kg/day HQ administration in male F344 rats, multivariate analysis showed a significant increase ($p < 0.05$) in combined urinary parameters. None of the parameters was increased at 1 or 3 weeks, except for a transient increase in alkaline phosphatase at one week in the high dose group. Also, none of the marker enzymes were increased in female F344 or male Sprague-Dawley rats. These authors reported some histopathological evidence of dose-related tubular degeneration and regeneration and tubular inflammation in male, but not female F344 or male Sprague-Dawley rats. The changes in male F344 rats reached statistical significance at 50 mg/kg/day; however, there also appeared to be changes at 25 mg/kg/day that did not reach significance because of the variability between animals. Male F344 rats administered 50 mg/kg/day HQ for 6 weeks showed significantly increased rates of cell proliferation in tubule cells of P1 and P2, but not P3 segments. Small increases at lower doses were not statistically significant, and only 50 mg/kg/day reached statistical significance for all segments combined (Figure 2). In the male F344, at 1 and 3 weeks and at lower doses, and in female F344 or Sprague-Dawley males, no cell proliferation increase was found with the possible exception of a transient increase in P2 cell proliferation in female F344 rats at 3 weeks. Evidence of a lack of HQ-enhanced hyaline droplet formation was reported in this study.

Nephrotoxicity of HQ-Glutathione Conjugates

Reaction of BQ with glutathione was found to produce multiconjugated HQ. Nephrotoxicity of these mono-, di-, and tri-substituted glutathione conjugates was studied by intravenous injection in male Sprague-Dawley rats (Lau et al., 1988). Twenty-four hours after injection of 5 to 250 $\mu\text{mol/kg}$ of the conjugates, blood was tested for serum glutamate pyruvate transaminase (SGPT) and blood urea nitrogen (BUN). Di-glutathiones were found to increase the BUN 3-4-fold more than HQ or monoconjugated HQ. Tri-conjugated HQ increased the BUN eight times more than HQ; the dose required to cause a significant elevation of BUN was 20 $\mu\text{mol/kg}$ (20.5 mg/kg). These authors also found that acivicin, which inhibits γ -GT, was capable of completely blocking the increased BUN induced by the conjugated HQ. In contrast, aminooxyacetic acid, the β -lyase inhibitor and probenecid, the organic anion transport inhibitor had no effect on the BUN increase. None of these conjugates increased the liver enzyme (SGPT). Histopathological evaluation found severe necrosis at the cortico-medullary junction, and these authors ascribed the S₃ segment of the proximal tubules as the site of toxicity.

Additional studies *in vitro* by these authors have implicated the brush border and mitochondria as target organelles in renal tubule cells (Hill et al., 1990, 1992). Studies of a renal proximal tubule derived cell line showed that triglutathione conjugated HQ-induced toxicity, determined by lactic dehydrogenase leakage, was accompanied by dose-dependent appearance in the culture medium of the membrane bound enzyme γ -GT. The triglutathione conjugate of HQ also inhibited mitochondrial respiration.

ANALYSIS OF MECHANISTIC STUDIES INVOLVING KIDNEY

The only test reported for HQ-DNA interaction in kidney did not show any adduct formation *in vivo* in spite of the relatively high HQ dose employed (English et al., 1994a). Although negative studies are sometimes difficult to interpret, availability of HQ-DNA and BQ-DNA adducts from *in vitro* reactions, used as positive controls, enhances the certainty of this negative *in vivo* finding.

There is a preliminary indication that HQ acts by tumor promotion in the kidney. Such a mechanism may involve cell damage and enhanced cell proliferation. HQ produced

renal tubular cell nephrotoxicity in male and female F344 rats when administered at 400 mg/kg acutely, but had little or no effect in male Sprague-Dawley rats (Boatman et al., 1992). When administered for 6 weeks, nephrotoxicity was found at 50 mg/kg/day in male F344 rats, but not in female F344 rats or in Sprague-Dawley rats (English et al., 1994b). In this study, enhanced cell proliferation was also found only in male F344 rats at six weeks but not at one or three weeks following HQ administration. This delay, in contrast to p-dichlorobenzene (Umemura et al., 1992), suggests that HQ does not produce a direct mitogenic effect, but that there is enhanced cell proliferation in response to nephrotoxicity. The lack of a mitogenic response in the study by Okazaki et al. (1993) is also consistent with this hypothesis.

One possible mechanism of nephrotoxicity is chemically-induced α_{2u} -globulin nephropathy, evident by hyaline droplet formation, that has been found to cause renal tumors only in male rats. However, the renal tubular cell adenomas in male rats, found in the bioassay, were not considered to be related to hyaline droplet nephropathy by the authors (NTP, 1989). English et al. (1994b) also investigated this mechanism utilizing techniques that have produced enhanced hyaline droplets for other chemicals, but no HQ-induced hyaline droplets were found. Also against involvement of α_{2u} -globulin nephropathy is an independent review of photographs of the renal lesions (G. Hard, personal communication). The finding of renal tumors primarily in the outer stripe of the medulla with two lesions deep in Zone 2 argues against α_{2u} -globulin nephropathy, which is found predominantly in the cortex (Zone 1). Zone 2 HQ-induced nephropathy would correspond to the P₃ segment of the tubule. Additionally, findings of renal tubular hyperplasia and adenomas in male mice in the Shibata et al. (1991) study indicate a mechanism not related to α_{2u} -globulin nephropathy.

Several mechanisms of toxicity have been proposed for development of renal tubule toxicity by chemicals (Lock, 1989; Anders, 1990; Vamvakas and Anders, 1990; Monks et al., 1990; Hard et al., 1993). These mechanisms involve metabolism of the chemical to an active form that may then be selectively reabsorbed to produce its toxic effect.

For a number of chemicals, glutathione conjugates have been implicated in renal toxicity via several different proposed mechanisms. The conjugates, formed in liver, are filtered by the renal glomerulus into the nephron where they may be reabsorbed by kidney

tubule cells. For vicinal dihaloalkanes, it has been proposed that nephrotoxicity proceeds from the glutathione conjugate by formation of an episulfonium ion, which can react with macromolecules including DNA. Such a mechanism does not pertain to HQ, however, as an episulfonium ion would not be formed from its corresponding glutathione conjugate.

Renal toxicity within the P₃ tubule segment has, in the case of hexachloro-1,3-butadiene (HCBD), been demonstrated to involve enzymatic cleavage of the glutathione conjugate by γ -GT and dipeptidases, to a cysteine conjugate (Lock and Ishmael, 1985). HCBD, its glutathione conjugate, cysteine conjugate, and N-acetylcysteine conjugate (mercapturic acid) all produce necrosis of the pars recta of the proximal renal tubule in the rat. Renal uptake and toxicity of HCBD and its derivatives could be inhibited by prior administration of probenecid, a competitive inhibitor of organic acid transport.

In the case of HCBD and other haloalkenes, the mechanism of glutathione-conjugate mediated renal toxicity involves β -lyase, located in tubular cells, which can cleave the carbon-sulfur bond of the cysteine conjugate (a cleavage product of the glutathione conjugate). This results in unstable thiols that are able to react with macromolecules. The thio derivative of HQ, however, does not share the instability characteristic of the analogous haloalkene thiols. Studies reported for 2-bromohydroquinone have shown that inhibition of β -lyase with aminooxyacetic acid had only minor effects on nephrotoxicity induced by this agent (Monks et al., 1988). Consequently, these authors have proposed that the β -lyase mechanism is not operative for hydroquinones. Also against involvement of the β -lyase mechanism, which has been linked to the P₃ segment tubular cells, are the findings that HQ-related renal tumors also appear to arise in P₁ and P₂ segment cells. Additionally, HQ-induced enhancement of cell proliferation was not found primarily in P₃ segment cells, but in P₁ and P₂ and only possibly in P₃ renal tubular cells.

Glutathione conjugates of HQ have been studied in male Sprague-Dawley rats and found to produce renal tubular cell toxicity that was prevented by acivicin, the γ -GT inhibitor (Lau et al., 1988). The triglutathione conjugate was the most toxic of those studied. Consequently, a role for this conjugate in renal toxicity is plausible. However, the finding that acute renal toxic effects were greater in female F344 rats than in male F344 rats (Boatman et al., 1992) requires explanation since renal tumors were produced only in male

rats. One explanation is the three times greater rate of cell proliferation in renal tubular cells of control males compared to females, which was found to be further enhanced by HQ-mediated toxicity (English et al., 1994b). Another complication is that Boatman et al. (1992) did not find HQ-induced renal toxicity in male Sprague-Dawley rats; therefore the relevance of studies of glutathione conjugates to HQ-related effects and tumorigenicity in the rat is open to question.

An alternative metabolic pathway for HQ is N-acetylation of the cysteine conjugate, which may result in urinary excretion. Such a metabolite has been reported in urine from HQ administration to male Sprague-Dawley rats by Nerland and Pierce (1990), and in male and female F344 rats by English et al. (1991). A potential mechanism for nephrotoxicity has been proposed involving a complex inter-organ transport system whereby the glutathione conjugate, after processing in intestine, liver and bile duct, becomes the N-acetylcysteine HQ conjugate. Transport of such N-acetylcysteine conjugates into the renal tubular cell has been proposed, involving the organic anion transport system (Monks et al., 1990). It is not known whether probenecid, which should inhibit this pathway, has a protective effect on HQ-induced nephrotoxicity.

Monks et al. (1990) have suggested that glutathione conjugates of HQ serve as transport and targeting metabolites. Since these HQ-conjugates arise from aromatic substitution, they are not candidates for the reversible glutathione transport mechanism, demonstrated for organic thiocyanates, which can release the parent compound in distant target organs. Thus, they suggest that the HQ-glutathione conjugate exerts its nephrotoxic action by redox cycling of the intact conjugate in the kidney. Spontaneous autoxidation of HQ to BQ has been shown chemically to be enhanced approximately eight-fold by HQ binding to glutathione compared to HQ alone (Brunmark and Cadenas, 1988). Lau et al. (1988) found that the degree of glutathione substitution increased the nephrotoxicity, and Puckett-Vaughn et al. (1993) have demonstrated that increasing substitution of HQ-glutathione conjugates decreased the thermodynamic oxidation potential, which may enhance redox cycling. A single high dose administration of HQ in male rats did not produce evidence of lipid peroxidation in the kidney (Okazaki et al., 1993), whereas evidence was found in the urine (Ekström et al., 1988).

An important finding with mechanistic implications is the possible role of spontaneous progressive nephropathy in the genesis of HQ-related renal tumors. The pathogenesis of this condition has been described in several reviews (Gray, 1977; Barthold, 1979; Goldstein et al., 1988). This condition occurs primarily in male rats and has been found to be enhanced by other chemicals such as those producing α_2 -globulin nephropathy. In the six-week study by English et al. (1994b), F344 females were not found to have any nephrotoxicity compared to F344 males; consequently, a sex-related mechanism may be involved for the induction of nephrotoxicity in male rats by HQ. Although there is no direct evidence of involvement of spontaneous progressive nephropathy in tumorigenicity, it is a process that has been shown to result in enhanced proliferation of tubular cells, which appears to be related to degree of nephropathy, and tumor promotion may result (Konishi and Ward, 1989). Enhancement of cell proliferation, associated with this nephropathy, as measured by increased labeling indices, occurs in both cortex and medulla, which is similar to the studies for HQ.

The severity of spontaneous progressive nephropathy has been found to be enhanced by HQ. Almost all renal adenomas in the NTP study were found to arise in rats that had an advanced form of this nephropathy. The tumors were histologically found to arise within foci of the nephropathy. Consequently, the causal role of spontaneous progressive nephropathy in HQ tumorigenicity may be a potentially fruitful area of future research.

STUDIES OF MECHANISMS NOT SPECIFIC TO RENAL TUMORS

Most of the genotoxic mechanistic studies of HQ-induced damage have centered on the hypothetical role of HQ in benzene-induced neoplasia. These genotoxicity studies of HQ are summarized in Table 5. Because both HQ and phenol have been identified as metabolites of benzene that accumulate in bone marrow, numerous studies have been published involving bone marrow, and in many instances, HQ and phenol have been tested together. For example, Table 6 shows *in vivo* adduct testing when HQ and phenol were co-administered.

Intravenous administration of radiolabeled HQ has resulted in radiolabel accumulation in macromolecules of rat bone marrow, but not liver or thymus (Greenlee et al., 1981a). It has been hypothesized that HQ binding both to proteins and DNA occurs following activation by a peroxidase of the type found in bone marrow, where HQ is oxidized to the more

reactive BQ or a semiquinone radical. It is possible that accumulation of HQ in bone marrow is due to this organ specific myeloperoxidase mechanism. Phenol has been found to enhance the formation of BQ from HQ by myeloperoxidase. BQ can be converted back to HQ in bone marrow and in other tissues containing quinone reductases and this reduction may play a protective role (Monks et al., 1992).

DNA Interaction with HQ

DNA interaction tests *in vitro*, including tests for DNA binding, strand breaks, repair, and inhibition of DNA replication, have shown largely positive results for HQ. Two tests reported HQ-DNA adduct formation *in vitro* as determined by ³²P-postlabeling. In contrast, as described previously, measurement in kidney did not reveal any *in vivo* adducts formed by HQ.

By directly reacting HQ and BQ with calf thymus DNA, two adducts including one identified as (3'-OH)benzetheno-(1,N²)deoxyguanosine have been found and characterized by Jowa et al. (1990). Levay et al. (1991) found that DNA adducts from an HQ reaction with calf thymus DNA were not the same as the adduct formed by HQ and BQ in bone marrow cells. A significant increase in peroxidase activity was associated with HQ-DNA adduct formation (Levay et al., 1993). Reddy et al. (1990) found DNA adducts, using ³²P postlabeling, in cultured rat Zymbal glands incubated for 48 hours with either phenol or HQ. HQ produced approximately 12 times as many adducts as phenol. These investigators also produced adducts by reacting BQ directly with rat kidney DNA for comparison to the Zymbal gland study.

Levay et al. (1991) reported DNA adduct formation by HQ in human bone marrow HL60 cells using ³²P-postlabeling. For both HQ and BQ, a single DNA adduct was found at levels of 0.05 to 10 adducts per 10⁷ nucleotides. DNA adducts detected after treatment of cells with either BQ or HQ migrated to the same location on TLC plates, indicating that the adducts were the same. They found that adduct levels were related to treatment time and concentration of HQ and BQ.

HQ doubled the steady state concentration of 8-hydroxy-2'-deoxyguanosine (8OHdG) *in vitro* in HL60 cells after a 30 min incubation (Kolachana et al., 1993). However, when

75 mg/kg HQ was administered intraperitoneally to male B6C3F₁ mice, analysis of bone marrow did not show an increase in 8OHdG. Statistically significant increases of this DNA adduct formed when HQ was co-administered with either 75 mg/kg phenol or catechol each given intraperitoneally.

Other tests of DNA interaction *in vitro* have yielded mixed results. No single strand breaks were induced by HQ in mouse lymphoma cells (Pellack-Walker and Blumer, 1986), but strand breaks were found in ϕ X-174 DNA (Lewis et al., 1988) and Chinese hamster bone marrow cells (Shimada et al., 1989). DNA synthesis was inhibited by HQ in mouse lymphoma cells (Pellack-Walker et al., 1985), rabbit bone marrow mitochondria (Schwartz et al., 1985) and mouse bone marrow cells (Lee et al., 1989). This inhibition of DNA synthesis was found to be irreversible in HeLa cells (Painter and Howard, 1982). There was no DNA synthesis inhibition by HQ in rat liver mitochondria (Schwartz et al., 1985).

Mutagenicity

Tests for HQ bacterial mutagenicity were mostly negative, but there were three positive results in 14 tests using *Salmonella typhimurium*. The positive results were obtained in two of three tests using TA1535 with no S9 activation, and in one of six tests using TA100 with S9 activation (Koike, 1988). Koike's positive test result was obtained in a fluctuation assay. *E. coli* testing was negative with and without S9 activation (Rossman et al., 1989). There was a single positive test in *S. cerevisiae* without activation (Cotruvo et al., 1977). Forward mutation tests in mouse lymphoma cells (McGregor et al., 1988) and Chinese hamster V79 cells (Glatt et al., 1989) were positive without activation. The sex-linked recessive lethal test in *Drosophila* was negative in two tests (Wild, et al., 1979; Gocke et al., 1981), and other *in vivo* tests for mutagenicity such as the spot test and dominant lethal assay were negative.

Cytogenicity

The most consistent genotoxic effect of HQ has been induction of chromosomal aberrations and aneuploidy. There were 16 positive *in vivo* and *in vitro* tests for chromosomal aberrations, cell division abnormalities and aneuploidy. Positive *in vivo*

chromosomal aberration and aneuploidy tests were found in male and female mouse bone marrow cells and in male mouse spermatocytes. There was also a single positive mitotic abnormality test in golden hamster bone marrow cells. Positive *in vitro* chromosomal aberration test results were also found in yeast, fungus, Chinese hamster DON and LUC cells, and in *Chara Zeylancia*.

The micronucleus test, usually an *in vivo* method to detect aberrant chromosome formation, was positive in all 12 *in vivo* tests reviewed. There were 11 micronucleus tests in bone marrow cells of male and female mice and, also, a test in fetal liver cells of male and female mice. There was a single *in vitro* micronucleus test in hamster cells, which was reported as negative.

Sister chromatid exchange tests in human lymphocytes *in vitro* were also found to be positive. In studies by Morimoto and Wolff (1980), Morimoto et al. (1983) and Erexson et al. (1985), testing was done with lymphocytes from single healthy donors. Knadle (1985) tested lymphocytes from six individuals and found a positive response in only three cases. Although the sample size was small, this might indicate that response to HQ may vary between individuals.

DNA Interaction Studies Using HQ and Phenol

Table 6 shows DNA adduct results *in vivo* when HQ was co-administered with phenol. These tests were performed because of the theory that phenol stimulates peroxidase-dependent metabolism of HQ (Eastmond et al, 1987; Barale et al., 1990). Hedli et al. (1989, 1990) measured HQ-DNA adducts from male Sprague Dawley rat liver and bone marrow, using ³²P postlabeling, following intraperitoneal dosing (50, 75, and 100 mg/kg each of HQ and phenol administered together). There was one major adduct, and two minor adducts, all of which were isolated and characterized using TLC. The major adduct co-chromatographed with an adduct synthesized from HQ and deoxyguanosine; however, the adduct was not identified.

DNA adducts were also found with combined HQ and phenol in female Sprague Dawley rat Zymbal gland *in vitro* but not *in vivo* (Reddy et al., 1990). In the *in vivo* study, a four day dietary regimen was used with 75 mg/kg/day each of HQ and phenol administered

together. Other attempts by Reddy et al. to find DNA adducts *in vivo* using the same protocol were negative in bone marrow, liver and spleen. It is possible that the four day feeding protocol was not long enough to allow DNA adducts to form, and that Hedli et al. were able to find adducts within four days because of the intraperitoneal exposure route.

Studies Relating HQ and BQ Genotoxicity

Evidence for the greater reactivity of BQ compared to HQ was provided by Pellack-Walker et al. (1985) who found that BQ inhibited DNA synthesis in mouse lymphoma L5178YS cells at much lower concentrations than those required by HQ or benzene. Cells were exposed to benzene, HQ and BQ at concentrations from 10^{-7} to 10^{-2} M. DNA synthesis was inhibited in the absence of discernable effects on cell membrane integrity and protein synthesis; therefore the effect was believed to be "DNA specific" by the investigators. Using the same cell line, Pellack-Walker and Blumer (1986) found that HQ was unable to induce DNA single strand breaks but that BQ produced dose-related breaks.

Lee et al. (1989) found that BQ was able to bind to available sulfhydryl (SH) groups on DNA polymerase α , and thus inhibit DNA synthesis. Schlosser et al. (1990) oxidized HQ to BQ by prostaglandin H synthase in the presence of arachidonic acid or H_2O_2 , as measured by HPLC and reductive electrochemistry. The resultant BQ formed a thio-protein adduct in the presence of cysteine, and also bound irreversibly to DNA. DNA binding was time dependent and did not occur in the presence of heat inactivated enzyme. The authors suggest that activation of HQ to BQ by prostaglandin H synthase represents a possible mechanism for binding to both protein and DNA.

Mechanistic Studies in Bone Marrow

Inhibition of microtubule assembly has been suggested as one of the damaging effects of macromolecular binding of HQ. Spindle impairment (due to inhibited microtubule assembly) was proposed as a possible route of HQ-induced chromosome damage by Irons and Neptun (1980). They found that HQ inhibited microtubule polymerization, and accelerated decay of tubulin-colchicine binding activity. Microtubule integrity is required for spindle formation during cell division. The authors concluded that HQ may interfere with

microtubule function, nucleotide binding or both. Adler and Kliesch (1990) evaluated micronucleus response in male and female mice with intraperitoneal HQ administration. The response was dose related and there was no gender difference. They found that time of appearance of micronuclei suggested that HQ had a spindle-inhibiting effect that resulted in lagging chromosomes. Miller and Adler (1989) concurred, proposing that HQ was a spindle inhibitor, demonstrated by induction of colchicine-like mitotic effects in mouse bone marrow. Corroboration for microtubule dysfunction induced by HQ, which has been oxidized to BQ, comes from an *in vitro* experiment by Epe et al. (1989) using α - and β -tubulin binding studies. Peroxidative metabolites of HQ reacted with tubulin, causing an inhibition of microtubule formation, which was more pronounced than that caused by HQ alone. The effect was not seen when BQ was scavenged from the incubation mixture by the addition of glutathione.

Thomas et al. (1990) reported that bone marrow derived macrophages, exposed to HQ or BQ, secreted less interleukin-1, a monokine capable of regulating synthesis of several hematopoietic factors, suggesting that HQ may interfere with hematopoiesis. Kalf et al. (1990) hypothesized that HQ was not toxic in liver, but that it was transported to bone marrow where it was metabolized to its bioreactive form by a peroxidase-mediated reaction. They proposed the following mechanism by which HQ was oxidized to the bioreactive BQ in bone marrow. Bone marrow macrophages contain considerable amounts of prostaglandin H synthase, an enzyme with cyclooxygenase and peroxidase activities. The sequential action of cyclooxygenase and hydroperoxidase converts arachidonic acid in marrow stromal cells to prostaglandins. Concurrently, the hydroperoxidase oxidizes HQ to the more reactive BQ, which in turn binds to macromolecules.

Twerdok and Trush (1990) investigated biochemical mechanisms of bone marrow toxicity induced by HQ and BQ. Non-cytotoxic doses of HQ impaired the ability of bone marrow stromal cells from DBA/2 mice to support granulocyte/macrophage colony formation *in vitro* by approximately 50%. When bone marrow stromal cells were pretreated with 1,2-dithiole-3-thione, which induces quinone reductase, the cells were protected from HQ-induced toxicity, as measured by increased cell survival (Twerdok et al., 1992). Dicumarol, which inhibits quinone reductase activity, was found to enhance HQ-induced toxicity. These

results indicate the necessity of oxidizing HQ to BQ before it can function as a cell toxin. The protective effect of induction of quinone reductase has also been found to be operative *in vivo* (Twerdok et al., 1993).

The effect of *in vitro* HQ pretreatment on the colony forming response of mouse bone marrow cells, stimulated with recombinant granulocyte/macrophage colony-stimulating factor, was examined by Irons et al. (1992). HQ, but not phenol, catechol or t,t-muconaldehyde enhanced growth factor response, evident as a 1.5- to 4.6-fold enhancement in colonies formed. The authors thought that the ability of HQ to alter intrinsic growth factor response and induce differentiation in a myeloid progenitor cell population might be important in pathogenesis of acute benzene-induced myelogenous leukemia. According to the authors, mechanisms to explain HQ-induced enhanced response to recombinant granulocyte/macrophage colony-stimulating factor include alterations in receptor-ligand affinity via direct binding to SH groups in the receptor complex, or direct effects on signal transduction or gene expression.

Analysis of Mechanisms Related to Bone Marrow Cells

It has been postulated that HQ is transported to bone marrow where it is oxidized by myeloperoxidase to bioreactive BQ, which then reacts with critical macromolecules such as protein and DNA (Kalf et al., 1990). However, there are conflicting test results regarding the ability of HQ to interact directly with DNA. In two tests, HQ-DNA adducts were formed in tissue culture of rat Zymbal glands or human bone marrow cells. These cells contain myeloperoxidase, therefore semiquinone or BQ may have been intermediates in the DNA reaction. DNA adduct formation *in vivo* was only reported when HQ and phenol were administered in combination, and this effect appeared to be related to administration route. Reddy et al. (1990) found no adducts when HQ and phenol were administered in diet, but Hedli et al. (1990) injected HQ and phenol intraperitoneally and were able to produce *in vivo* adducts in liver and bone marrow.

Mutagenicity test findings suggest that HQ interaction with DNA, which is required for positive results in bacterial mutation tests, generally did not occur in these systems, and that the rat liver S9 fraction was not able to activate HQ to a reactive species. Since positive

results were obtained with yeast and mammalian cells *in vitro*, it is possible that HQ could be activated to a reactive species only in these systems. Data suggest that HQ activating factors, such as oxidizing agents that promote the spontaneous conversion of HQ to BQ, might be present in some systems and absent in others. It is also possible that DNA binding occurs only in certain species, organs and tissues, or that binding to protein may be occurring preferentially to DNA binding.

The battery of mostly positive cytogenetic tests indicates that HQ is able to cause chromosomal damage in fungus, mouse, hamster and human cells. Positive results in mammalian cytogenetic testing were found in the following tissues that were studied: bone marrow, spermatocytes, lymphocytes and fetal liver. The positive micronucleus test in bone marrow and fetal liver cells suggests lagging chromosome formation as the damaging event. Positive cytogenetic test results, compared to mostly negative results seen in mutagenicity testing, suggest that the mechanism responsible for HQ-induced chromosomal damage does not involve DNA adduct formation.

Specificity for bone marrow seen in the positive micronucleus data, and lack of positive response in Ames testing indicate that enzymes present in bone marrow and not in rat liver, such as myeloperoxidase, might be activating factors. Metabolism studies have shown that HQ is oxidized to BQ and that BQ is the more reactive species. Although the mechanism by which HQ induces micronuclei and aneuploidy is not entirely clear, it has been suggested that spindle inhibition, which induces lagging chromosomes, is the most likely cause. Spindle inhibition may be caused by binding of HQ to SH groups on spindle tubulin as suggested by Irons and Neptun (1980). This mechanism may also be involved in the findings of other *in vitro* tests. For example, as shown in Table 5, HQ produced inhibition of DNA synthesis in mouse lymphoma cells, but no DNA strand breaks. Consequently, DNA synthesis inhibition does not appear to depend on direct DNA damage, but rather on interference with the replication process, which may be due to HQ binding the SH groups on cysteine in polymerase α as suggested by Lee et al. (1989).

The relevance of chromosomal damage *in vivo* and *in vitro* to cancer mechanism is unclear, but chromosomal damage and fragmentation are typical of human leukemia. Micronuclei and SCE induction, the main tests showing HQ effects, may be a cause, effect

or unrelated side effect of the tumorigenic process. It should be noted that HQ did not produce an increase in hematopoietic neoplasia in mice, which is the only species used in the micronucleus test. Also, the available evidence from the two bioassays for HQ does not establish that HQ produces mononuclear cell leukemia in rats.

Relevance of Studies Aimed at Benzene Effects

As described previously, many studies of HQ have been initiated to investigate effects of benzene, which is known to be metabolized to HQ. However, results of the bioassays show that the neoplastic effects of benzene are very different from those described for HQ. For example, the NTP reported results of studies with benzene in F344 rats and B6C3F₁ mice (NTP, 1986). Neoplasia was found in the Zymbal gland, and papillomas and squamous cell carcinomas were found in the oral cavity in male and female rats, and in the skin of male rats. In female mice, there were tumors of Zymbal gland, lung, Harderian gland, mammary gland (females) and preputial gland (males). There were no reported increases of renal adenomas in the benzene studies. Consequently, it would appear that target organs for benzene and HQ are completely different. This is especially noteworthy for the NTP studies since the same strain and species were used, and these studies were performed within approximately the same time frame.

In spite of great interest in the genotoxic potential of HQ, relevance of these findings to neoplasms produced by HQ remains in question. No DNA adducts have been found in the F344 male rat kidney, which is the only clearly demonstrated target organ for neoplasia. Although DNA adducts have been produced *in vitro* in Zymbal gland and bone marrow cells, these tests were not performed *in vivo* using HQ alone, since these studies were designed to mimic benzene metabolites. Finding *in vivo* DNA adducts in liver and bone marrow by Hedli et al. (1990) using a combination of HQ and phenol does not indicate DNA reactivity when HQ is administered alone. Consistent positive findings in other tests of genotoxicity have included chromosomal aberrations, aneuploidy and micronucleus tests. However, it appears that these studies may best be explained by epigenetic effects rather than DNA reaction. It is known that HQ interferes with microtubulin function by compromising protein sulfhydryl groups, and this may be the cause of these positive genotoxicity tests.

HUMAN EXPOSURE STUDIES

Few studies have investigated the potential effects of HQ exposure in humans. A study of persons employed as photographic processors was reported by Frielander et al. (1982). However, no significant exposures to HQ in this group of workers was identified. A mortality study of employees engaged in the manufacture and use of HQ has been reported by Pifer et al. (1994), which provides the most comprehensive epidemiological investigation of HQ. A previous report from this chemical plant, which included persons exposed to HQ or other chemicals was published by Pifer et al. (1986) with similar results. Death certificates in a cohort of 858 men and 21 women were compared to a group of occupational referents and to mortality data for the general population of the state. At the plant, historical HQ levels over the 61 years of production were estimated to be at or above 2 mg/m^3 for 37 years including a 21 year period at or above 5 mg/m^3 . Also, there was a concurrent BQ exposure as part of the production process, which ranged from about 0.2 to 1 mg/m^3 . The mean years of employment were 13.7 and mean follow-up from first exposure was 26.8 years. The cohort showed a statistically significant deficit in total and cancer mortality. No statistically significant increase was reported for renal cancer (2 observed vs. 1.3 expected), and there were no deaths reported due to liver cancer or leukemia.

The major potential occupational exposure pathway of HQ likely for humans is dermal exposure to HQ in photographic processing operations. For dermal absorption, there are two reports that give some indication of potential human intake. Bucks et al. (1988) administered radiolabeled HQ in an alcohol-water solution to the skin of the human forehead. Urine was collected for 24 hours. The average dermal absorption determined by excretion analysis was 35% of the 20 mg amount of HQ applied to 16 cm^2 of skin for 24 hours. In another study, human stratum corneum was exposed for one hour in a diffusion cell to a 5% aqueous solution of HQ, which is typical of the concentration used in photographic processing (Barber et al., 1993). The absorption rate was found to be approximately $0.5 \text{ } \mu\text{g/cm}^2/\text{hr}$. This rate is comparable to the rate that can be calculated from the Bucks et al. (1988) study.

FINDINGS, UNCERTAINTIES, AND RELEVANCE TO HUMAN EXPOSURE

Tables 1-4 present a summary of the available bioassay data. Our analysis of the evidence for tumorigenicity has been discussed previously and is summarized in Table 4. The only tumor type for which there is convincing evidence of tumorigenicity is the renal adenoma in the male rat. Not only is the evidence qualitatively consistent for renal tumors, but data from these combined studies result in a plausible dose-response curve (Figure 2). For the other benign tumor types reported to be statistically significantly increased in only one sex of one study, the contradictory data, the high incidence in controls, the lack of dose relationship, and lack of any increases in carcinogenicity lead us to conclude that there is a lack of evidence of carcinogenicity related to these tumors.

Studies in kidney do not implicate HQ in direct DNA adduct formation. Consequently, most recent research has been directed at investigating mechanisms capable of inducing toxicity, involving the transport of chemical species in the target renal tubule cell. Studies of the nephrotoxicity by which HQ may produce tumors in the kidney of male rats have not definitely established an underlying mechanism. Several proposed mechanisms for renal toxicity by other chemicals are not likely to be appropriate for HQ, including β -lyase catalyzed production of reactive thiols derived from the glutathione conjugate or reversible glutathione conjugation. However, there is good evidence that a glutathione conjugate may be involved, and two mechanisms of toxicity by this metabolic pathway can be proposed: one involving redox cycling of HQ and the corresponding quinone producing reactive free radical species, and the other involving covalent binding of activated HQ conjugates to critical protein target sites.

Glutathione conjugates have been implicated as transport forms to the kidney for a number of chemicals. The nephrotoxicity of BQ-glutathione conjugates has been blocked by acivicin, which is an irreversible inhibitor of γ -GT. It has been proposed that the nephrotoxicity of HQ-glutathione conjugates was associated with their selective uptake into renal cells after metabolism to the corresponding cysteine conjugates by γ -GT and dipeptidases. However, there is some doubt whether the strain of rat employed in these studies actually demonstrates HQ-induced renal toxicity. Consequently, the detailed

mechanism by which the glutathione conjugates of HQ produce nephrotoxicity in rats is still not understood.

Involvement of epigenetic mechanisms in the development of tumors has become increasingly evident (Williams and Weisburger, 1991). Involvement of enhanced cell proliferation, which can arise reactively to cell death, has been proposed as a mechanism of promotion of endogenously available initiated cells (Loeb, 1989; Cohen and Ellwein, 1990). It would appear that HQ-induced tumor formation in the male F344 rat kidney may involve such a mechanism; however, the exact features of the nephrotoxicity remain to be elucidated. Such a tumorigenic effect would not be operative at doses that do not produce nephrotoxicity and enhanced cell proliferation. If tumorigenicity is associated with the rat spontaneous progressive nephropathy, then the relevance of these benign tumors to humans is questionable.

HQ did not produce nephrotoxicity or enhanced cell proliferation at 2.5 mg/kg/day, which was ten times lower than the lower dose in the positive bioassay, which showed only a low incidence of the benign renal tumors and some evidence of nephrotoxicity in male F344 rats. The lack of significant renal findings at equivalent or higher doses in female rats and male Sprague-Dawley rats indicates that male F344 rats are the most sensitive to the renal effects of low level or chronic HQ exposure of any animal tested. The calculated human absorption of about 0.005 mg/kg for a one hour direct exposure for both hands to an aqueous 5% solution of HQ would represent a reasonable worst case occupational exposure to HQ used in photographic processing. This dosage is 1/500 of the NOEL for cell proliferation and nephrotoxicity and 1/5,000 of the dose producing a small incidence of benign renal tumors in the male F344 rats. Consequently, occupation exposures in photographic processing should not pose a cancer risk in humans. The results of the recent epidemiology study of HQ production and use population (Pifer et al., 1994) also provides supporting evidence that other occupational exposures do not present a carcinogenic risk to humans.

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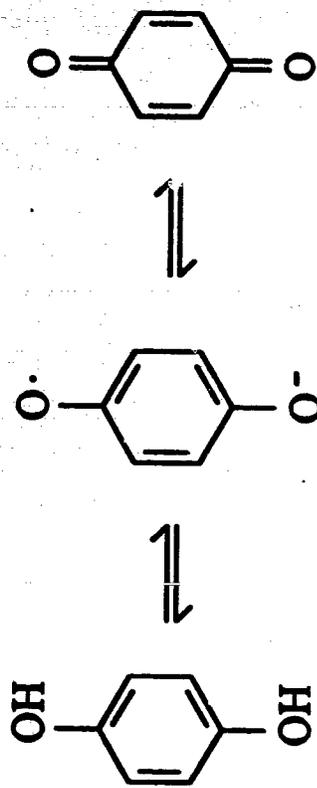
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Figure 1: Hydroquinone Oxidized to Benzoquinone.

Figure 2: Dose-Response of Hydroquinone in Male F344 Rats: ■, renal adenomas (NTP, 1989); □, renal adenomas (Shibata et al., 1991); ○, BrdU incorporation, ratio to controls. Dotted line connects data points between two different bioassays. Doses for NTP, 1989 and English et al., 1994b are 5 days/week by gavage; for Shibata et al., 1993 are daily dose in the diet, approximated.



Hydroquinone

Semiquinone

Benzoquinone

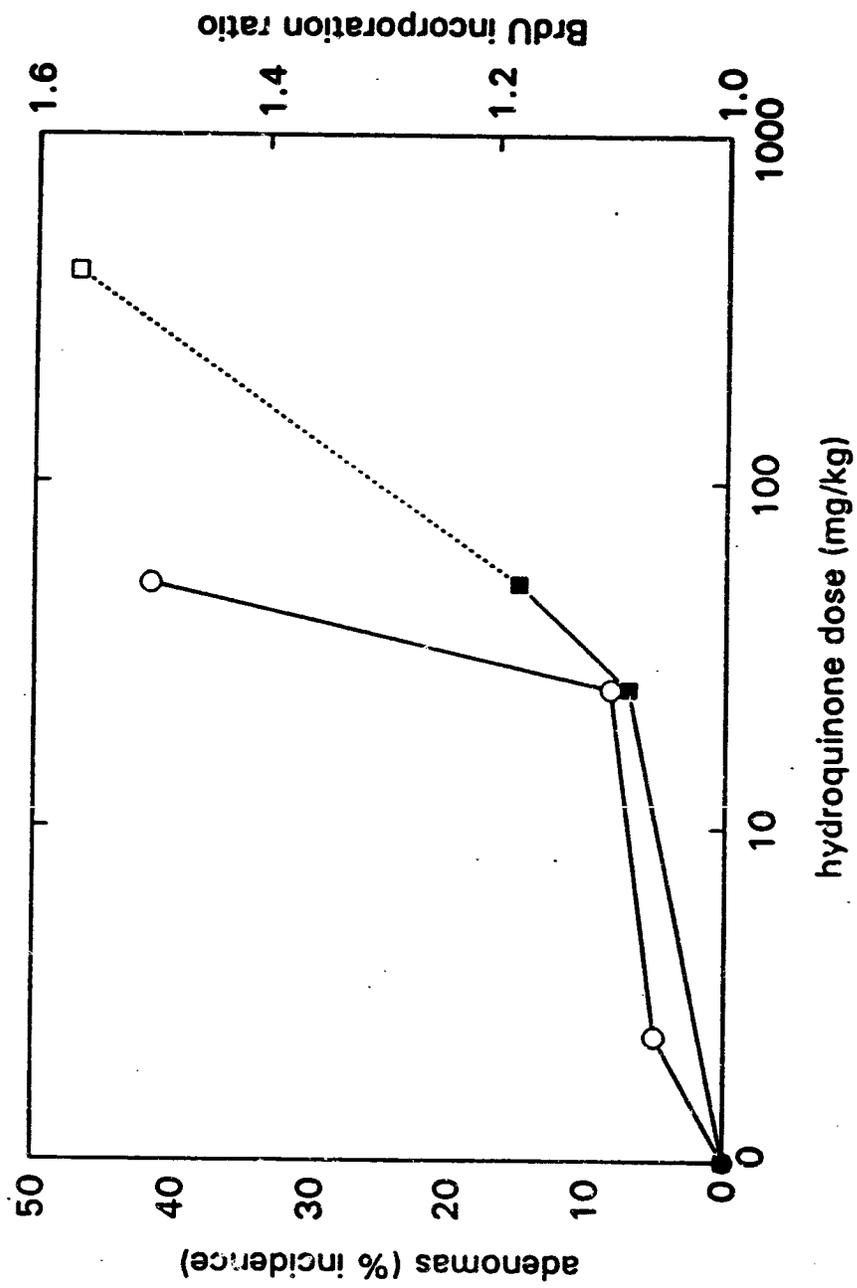


Table 1

Neoplasia Incidences for Hydroquinone in F344 Rats

Sex	Dose¹ (mg/kg/day)	Renal Adenoma	Mononuclear Leukemia in the Spleen²
Males³	0	0/55 (0%)	28/55 (51%)
	25	4/55 (7%)	26/52 (50%)
	50	8/55 (15%)⁴	31/55 (56%)
Males⁵	0	0/30 (0%)	6/30 (20%)
	350	14/30 (47%)	8/30 (27%)
Females³	0	0/55 (0%)	9/55 (16%)⁶
	25	0/55 (0%)	15/55 (27%)
	50	0/55 (0%)	22/55 (40%)⁴
Females⁵	0	0/30 (0%)	5/30 (17%)
	368	0/30 (0%)	4/30 (13%)

¹5 days per week in NTP study, 7 days per week in Shibata study

²Reported as leukemia or lymphoma by Shibata (personal communication)

³NTP (1989)

⁴Significantly different from control by Fisher Exact Test, $p < 0.05$

⁵Shibata et al. (1991)

⁶Historical range $25\% \pm 15\%$

Table 2
Hepatic Lesions for Hydroquinone B6C3F₁ Mice

NTP (1989)

Sex	Dose (mg/kg) 5 days/week	Basophilic Foci	Eosinophilic Foci	Adenomas	Hepatocellular Carcinomas	Adenomas or Hepatocellular Carcinomas
Male	0	2/55 (4%)	4/55 (7%)	9/55 (16%)	13/55 (24%)	20/55 (36%)
	50	5/54 (9%)	2/55 (4%)	21/54 (39%) ²	11/54 (20%)	29/54 (54%)
	100	11/55 (20%)	1/55 (2%)	20/55 (36%) ¹	7/55 (13%)	25/55 (45%)
Female	0	2/55 (4%)	1/55 (2%)	2/55 (4%)	1/55 (2%)	3/55 (5%)
	50	6/55 (11%)	2/55 (4%)	15/55 (27%) ³	2/55 (4%)	16/55 (29%) ³
	100	3/55 (5%)	0/55 (0%)	12/55 (22%) ²	2/55 (4%)	13/55 (24%) ²

¹Statistically significant increases in tumor incidence $p < 0.05$ by the Fisher Exact Test

²Statistically significant increases in tumor incidence $p < 0.01$ by the Fisher Exact Test

³Statistically significant increases in tumor incidence $p < 0.001$ by the Fisher Exact Test

Table 3
Hepatic Lesions for Hydroquinone
in B6C3F₁ Mice

Shibata et al. (1991)

Sex	Dose (mg/kg/day)	Altered Foci	Adenomas	Hepatocellular Carcinomas
Males	0	4/28 (14%)	6/28 (22%)	7/28 (26%)
	1046	14/30 (47%)	14/30 (47%) ¹	6/30 (20%)
Females	0	0/29 (0%)	0/29 (0%)	1/29 (3%)
	1486	2/30 (7%)	1/30 (3%)	0/30 (0%)

¹Statistically significant increase in tumor incidence $p < 0.05$ by the Fisher Exact Test

Table 4
Summary of the Presence of Significant Tumor Increases

	Male Rat Kidney Adenomas	Female Rat Mononuclear Leukemias	Male Mouse Liver Tumors	Female Mouse Liver Tumors
NTP (1989)	increased	increased	no change	increased adenomas
Shibata et al. (1991)	increased	no change	increased adenomas	no change
Evidence of Tumorigenicity	yes	no	equivocal ¹	equivocal

¹ AHF evaluations; see text for explanation.

TABLE 5. Genotoxicity Assays of Hydroquinone

Species/Strain	Cell/Strain	In Vivo	Test Results		References
			without activation	with activation	
DNA INTERACTION					
DNA Binding					
Human	Bone Marrow HL60 Cells	no ¹	+ ³	ND ²	Levy et al. (1991)
Rat/Sprague Dawley	Zymbal Gland	no	+	ND	Reddy et al. (1990)
Rat/F-344	Kidney	yes ⁴	.	NA ⁶	English et al. (1994a)
Tissue Binding					
Rat/Fischer 344	Bone Marrow Tissue	yes	+	NA	Greenlee et al. (1981)
Oxidative DNA Damage					
Human	Bone Marrow HL60 Cells	no	+	ND	Koilechana et al. (1993)
Mouse/B6C3F ₁	Bone Marrow Tissue	yes	.	NA	Koilechana et al. (1993)
Strand Break and Repair					
Mouse	Lymphoma Cells/L5178YS	no	.	ND	Pelleck-Walker & Blumer (1986)
φx-174 DNA		no	+	ND	Lewis et al. (1988)
Chinese Hamster	Bone Marrow Cells	no	ND	+	Shimada et al. (1989)
Inhibition of DNA Replication					
Human	HeLa Cells	no	+	+	Painter & Howard (1982)
Mouse	Lymphoma Cells/L5178YS	no	.	+	Pelleck-Walker et al. (1985)
Rat/Wistar	Liver Mitochondria	no	.	ND	Schwartz et al. (1985)
Rabbit/White New Zealand	Bone Marrow Mitochondria	no	+	ND	Schwartz et al. (1985)
Rat Liver Mitochondrial					
DNA Polymerase					
Mouse COBS CD-1 ICR BR	Bone Marrow Cells	no	+	ND	Schwartz et al. (1985)
		no	+	ND	Lee et al. (1989)

TABLE 5 -- Continued

Species/Strain	Cell/Strain	In Vivo	Test Results		References
			without activation	with activation	
MUTAGENICITY					
<u>Reverse Mutation</u>					
<i>S. typhimurium</i>	TA100	no	ND	-	Cotruvo et al. (1977)
<i>S. typhimurium</i>	TA100	no	ND	-	Epler et al. (1978)
<i>S. typhimurium</i>	TA98	no	-	-	Wild et al. (1979)
<i>S. typhimurium</i>	TA1535	no	+	-	Wild et al. (1979)
<i>S. typhimurium</i>	TA100	no	-	NL	Rapson et al. (1980)
<i>S. typhimurium</i>	TA98	no	-	-	Florin et al. (1980)
<i>S. typhimurium</i>	TA100	no	-	-	Florin et al. (1980)
<i>S. typhimurium</i>	TA1535	no	-	-	Florin et al. (1980)
<i>S. typhimurium</i>	TA1537	no	-	-	Florin et al. (1980)
<i>S. typhimurium</i>	TA1535A	no	+	-	Florin et al. (1980)
<i>S. typhimurium</i>	TA97	no	-	-	Gocke et al. (1981)
<i>S. typhimurium</i>	TA98	no	-	-	Sakai et al. (1985)
<i>S. typhimurium</i>	TA100	no	-	-	Sakai et al. (1985)
<i>S. typhimurium</i>	TA100	no	-	-	Sakai et al. (1985)
<i>S. typhimurium</i>	TA97	no	-	+	Sakai et al. (1985)
<i>S. typhimurium</i>	TA98	no	-	-	Koike et al. (1988)
<i>S. typhimurium</i>	TA100	no	-	-	Glatt et al. (1989)
<i>S. typhimurium</i>	TA102	no	-	-	
<i>S. typhimurium</i>	TA104	no	-	-	
<i>S. typhimurium</i>	TA98	no	-	-	
<i>S. typhimurium</i>	TA100	no	-	-	NTP (1989)
<i>S. typhimurium</i>	TA1535	no	-	-	
<i>S. typhimurium</i>	TA1537	no	-	-	
<i>E. coli</i>	WP2S (λ)	no	-	-	
<i>S. cerevisiae</i>	D3	no	+	ND	Rossmann et al. (1989) Cotruvo et al. (1977)
<u>Forward Mutation</u>					
Mouse	Lymphoma/L5178Y	no	+	ND	McGregor et al. (1988)
Chinese Hamster	V79	no	+	ND	Glatt et al. (1989)

TABLE 5 -- Continued

Species/Strain	Cell/Strain	In Vivo	without activation	Test Results with activation	References
Sex Linked, Recessive Lethal					
D. melanogaster		yes	-	NA	Wild et al. (1979)
D. melanogaster		yes	-	NA	Gocher et al. (1981)
Spot Test					
Mouse/C57BL/6JHAN		yes	-	NA	Gocher et al. 1983
Dominant Lethal					
Rat COBCD(SD)BR		yes	-	NA	Krasavage (1984)
CYTOGENICITY					
Chromosomal Aberrations					
Golden Hamster	Bone Marrow Cells	yes	+	NA	Parmentier (1953)
China Zeylanica/ Klein ex Willd		no	+	NA	Chatterjee & Sharma (1972)
Fungus Mouse/ (101/E1 x C3H/E1) F ₁	A. nidulans Strain 19 Bone Marrow Cells	yes	+	NA	R. Crebelli et al. (1987)
(102/E1 x 3H/E1) F ₁ Chinese Hamster	Bone Marrow Cells	yes	+	NA	Miller & Adler (1989)
Chinese Hamster	Bone Marrow Cells	yes	+	NA	Xu & Adler (1990)
Fungus Mouse/ (102/E1 x C3H/E1) F ₁	DON.Wg3H LUC 1 A. nidulans/P1 Spermatocytes Bone Marrow Cells	yes yes yes yes yes	+	NA	Parry et al. (1990)
		yes	+	NA	Parry et al. (1990)
		yes	+	NA	Crebelli et al. (1991)
		yes	+	NA	Ciranni & Adler (1991)
		yes	+	NA	Marrazzini et al. (1991)

TABLE 5 -- Continued

Species/Strain	Cell/Strain	In Vivo	Test Results without activation	Test Results with activation	References
<u>Aneuploidy</u>					
Fungus	A.nidulans	no	+	ND	Kappas (1990)
Yeast/D6		no	+	ND	Parry et al. (1990)
Mouse	Bone Marrow Cells	yes	+	NA	Marrazzini et al. (1991)
Mouse/ C57B1/Cne x C3H/Cne	Bone Marrow Cells	yes	+	NA	Pacchierotti et al. (1991)
<u>Micronucleus</u>					
Mouse	Bone Marrow Erythrocytes	yes	+	NA	Eckhardt et al. (1979)
Mouse/NMRI	Bone Marrow Cells	yes	+	NA	Gocke et al. (1981)
Mouse/NMRI	Bone Marrow Erythrocytes	yes	+	NA	Tunek et al. (1982)
Mouse/CD-1	Bone Marrow Erythrocytes	yes	+	NA	Gad-El-Karim et al. (1985)
Mouse/CD-1	Bone Marrow Erythrocytes	yes	+	NA	Gad-El-Karim et al. (1986)
Mouse/Swiss CD-1	Bone Marrow Cells	yes	+	NA	Ciranni et al. (1988)
Mouse/Swiss CD-1	Fetal Liver Cells	yes	+	NA	Ciranni et al. (1988)
Mouse/ ddY		yes	+	NA	Shimada et al. (1989)
Mouse/ (101/E1 x C3H/E1)F ₁	Bone Marrow Erythrocytes	yes	+	NA	Adler & Kliesch (1990)
Mouse/Swiss CD-1	Bone Marrow Erythrocytes	yes	+	NA	Barale et al. (1990)
Chinese Hamster	DON or LUC	no	-	ND	Parry et al. (1990)
Mouse	Bone Marrow Cells	yes	+	NA	Marrazzini et al. (1991)
Mouse/ C57B1/Cne x C3H/Cne	Bone Marrow Cells	yes	+	NA	Pacchierotti et al. (1991)

TABLE 5 -- Continued

Species/Strains	Cell/Strain	In Vivo	without activation	Test Results with activation	References
Sister Chromatid Exchange					
Human	Lymphocytes	no	-	ND	Morimoto & Wolff (1960)
Human	Lymphocytes	no	-	+	Morimoto et al. (1963)
Human	Lymphocytes	no	+	ND	Eronson et al. (1965)
Human	Lymphocytes	no	+	ND	Kashe (1965)
MISCELLANEOUS					
Inhibition of Microtubule Polymerization					
Rat/Fischer 344	Brain Microtubulin	no	+	ND	Irons & Nephew (1960)

¹no indicates test was not done in vivo

²+ indicates positive result

³ND indicates test was not done

⁴yes indicates test was done in vivo

⁵- indicates negative result

⁶NA indicates not applicable

⁷positive with ZLM but not Vogel-Bonner medium, TA1535A is not genetically characterized.

⁸using fluctuation assay

⁹this test was positive in 3 of 6 individuals

TABLE 6 -- Genotoxicity Assays of Hydroquinone Coadministered with Phenol

Species/Strain	Cell/Strain	In Vivo	without activation	Test Results with activation	References
DNA INTERACTION					
DNA Binding					
Rat/Sprague Dawley	Liver	yes ¹	+ ²	NA ³	Hedji et al. (1989)
Rat/Sprague Dawley	Bone Marrow	yes	+	NA	Hedji et al. (1990)
Rat/Sprague Dawley	Zymbal Gland	yes	⁴	NA	Reddy et al. (1990)
Rat/Sprague Dawley	Bone Marrow	yes	-	NA	Reddy et al. (1990)
Rat/Sprague Dawley	Liver	yes	-	NA	Reddy et al. (1990)
Rat/Sprague Dawley	Spleen	yes	-	NA	Reddy et al. (1990)
Oxidative DNA Damage					
Mouse/B6C3F ¹	Bone Marrow	yes	+	NA	Kolanchana et al. (1993)

¹yes indicates test was done in vivo
²+ indicates a positive result
³NA indicates test was not applicable
⁴- indicates a negative result