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THE EFFECTS OF BUTYLATED HYDROXYANISOLE AND BUTYLATED HYDROXYTOLUENE ON RENAL FUNCTION IN THE RAT

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THE EFFECTS OF BUTYLATED HYDROXYANISOLE AND BUTYLATED HYDROXYTOLUENE ON RENAL FUNCTION IN THE RAT

By

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ABSTRACT

THE EFFECTS OF BUTYLATED HYDROXYANISOLE AND BUTYLATED HYDROXYTOLUENE ON RENAL FUNCTION IN THE RAT

by

Sue Marie Ford

The effects of administration of the food antioxidants butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) on selected aspects of renal function were studied in male rats. Renal cortical slices from animals treated with 500 mg/kg BHA or BHT had diminished capacity to accumulate organic acid, but not base. Despite continued antioxidant administration, the transport capacity of slices for organic acids returned to control levels by the sixth dose. The effect of BHT treatment was of greater magnitude and duration than with BHA. No effect on renal transport capacity for organic acid was noted with phenobarbital pretreatment prior to BHT administration. Phenobarbital pretreatment diminished the increased liver and adrenal weight noted with BHT treatment, although stimulation of hepatic BHT oxidase and hexobarbital hydroxylase activities was greater.

Potassium, but not sodium, excretion in urine was proportional to intake in BHA-treated animals. Sodium and potassium balances were maintained in rats receiving BHT.

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INTRODUCTION

The kidneys are important in preserving homeostasis when the body is exposed to conditions which may disturb the internal environment. In addition to maintaining a constant composition of the body fluid compartments, they have an essential role in the elimination of potentially toxic foreign compounds. Any agent which impairs the renal capacity to perform these functions may have serious consequences for the health of the animal.

The kidneys of man receive a large proportion of the cardiac output (25%) relative to their size (0.5% of body weight) (1). During the process of excretion, the concentration of xenobiotics may increase in the nephron as the result of the reabsorption of water during the formation of urine, and possibly through the action of systems which actively transport various organic ions from the blood into the urine. These factors indicate that the kidneys are subject to significant exposure to chemicals which may be in the plasma.

The use of food additives has increased in the past decade, and it has become of increasing concern to ensure the safety and usefulness of all such substances (2). [Although the concentration of any particular additive in the diet may be low, chronic exposure of the kidneys to these compounds may have deleterious effects on renal function.]

Therefore, this study was undertaken in order to examine the effects of the synthetic antioxidants butylated hydroxyanisole and butylated hydroxytoluene on selected aspects of renal function.

Antioxidants in Foods

Unsaturated lipids are susceptible to a variety of degradative changes as the result of exposure to light, heat, oxygen, and trace metals (3). Because it is often impossible to suitably protect food products during processing, distribution, and storage, prevention of the deterioration of fats and oils by chemical means is of economic importance to the food industry. In order to be acceptable for use in foods such a chemical must have certain properties (4):

a) Effectiveness; that is, the ability to prevent deterioration, preferably when added in small quantities.

b) Carry-through; the ability to prevent deterioration in the final product.

c) No effect in the quantities used on the organoleptic qualities (odor, flavor, color) of foods.

d) Fat solubility.

e) No hazard to the consumer in the quantities used.

Many vegetable oils, particularly those from seeds, contain natural antioxidants such as vitamin E (3). These natural compounds do not possess sufficient stability to be useful in processed foods and much work has therefore been devoted to the development of synthetic antioxidants.

In the early part of this century, the increased use of commercially prepared foods prompted the testing and development of suitable antioxidants for those containing lard. In 1948 the American Meat Institute Foundation announced a new synthetic antioxidant, butylated hydroxyanisole (BHA) (4). BHA was found to be effective in stabilizing lard and increasing the shelf-life of products made with lard. In 1954, the Foundation introduced BHT (butylated hydroxytoluene) for use in foods (5). BHT was slightly more effective than BHA, but did not have comparable carry-through. However, the two antioxidants were found to be synergistic, and a mixture of 0.01% of each provided maximum stability. With the use of these compounds the shelf-life of some foods could be increased 15-200% (6).

Regulation of Use of BHA and BHT

Although BHA and BHT are substances which are added to foods, they are not legally classified as food additives by virtue of their inclusion on the Generally Regarded As Safe (GRAS) list (2). New additions to the list should be tested for usefulness and safety, yet a clause in the Food Additives Amendment of 1958 allowed for the admission of any substance in use prior to January 1, 1958 if there were no objections from the scientific community (2,7). Consequently BHT, which had been in use for only four years, and BHA were included on the list of GRAS substances despite published data available at that time suggesting possible adverse effects (8,9). This allowed for the use of these compounds in foods with only those limitations imposed by standard manufacturing practices (7). For products covered by the Meat Inspection Act of 1954 and the Poultry Inspection Act, the limits are 0.01%

of either antioxidant based on the fat content of the food, or 0.02% in total (6). For products covered by other regulations, the permissible limits range from 2 ppm for dessert dry mixes to 1000 ppm for active dry yeast and chewing gum base (6).

The World Health Organization (WHO) and Food and Agriculture Organization (FAO) met jointly in 1955 to consider the problems involved in the use of food additives. Starting in 1956, an FAO/WHO Expert Committee on Food Additives issued a series of annual reports reviewing the toxicity of various classes of additives, and setting guidelines for their use (7). As the outcome of its efforts in evaluating these compounds, the Committee attempted to establish Acceptable Daily Intake (ADI) zones of additives for humans. The ADI were divided into unconditional and conditional zones. The unconditional zones represent levels of the additives which the Committee felt could be ingested safely by humans (10). The conditional zones were established for those compounds for which not enough information was available to adopt a higher limit of intake (11).

Based on the toxicological data available in 1962, the Expert Committee recommended an unconditional ADI zone for BHA of 0.0-0.5 mg/kg/day, and a conditional zone of 0.5-2.0 mg/kg/day (11). The Committee felt that it did not have enough data to establish an unconditional zone for BHT; however, a conditional zone was set at 0.0-0.5 mg/kg/day. Comparison of the ADI zones with the average daily intake of the antioxidants in the U.S. (0.2 mg/kg/day) indicates that the current applications of BHA and BHT are within the recommended limits (6).

In 1972 a Select Committee of scientists in various disciplines was established by the FDA to review substances on the GRAS list (7); BHA and BHT are being evaluated in this process. The task of this Committee is to consider the compounds already on the list and to recommend the removal of those for which the data suggest a hazard to the population. Numerous papers have been published regarding BHA and BHT but the significance of many of the results--such as induction of hepatic enzymes--has yet to be determined (7).

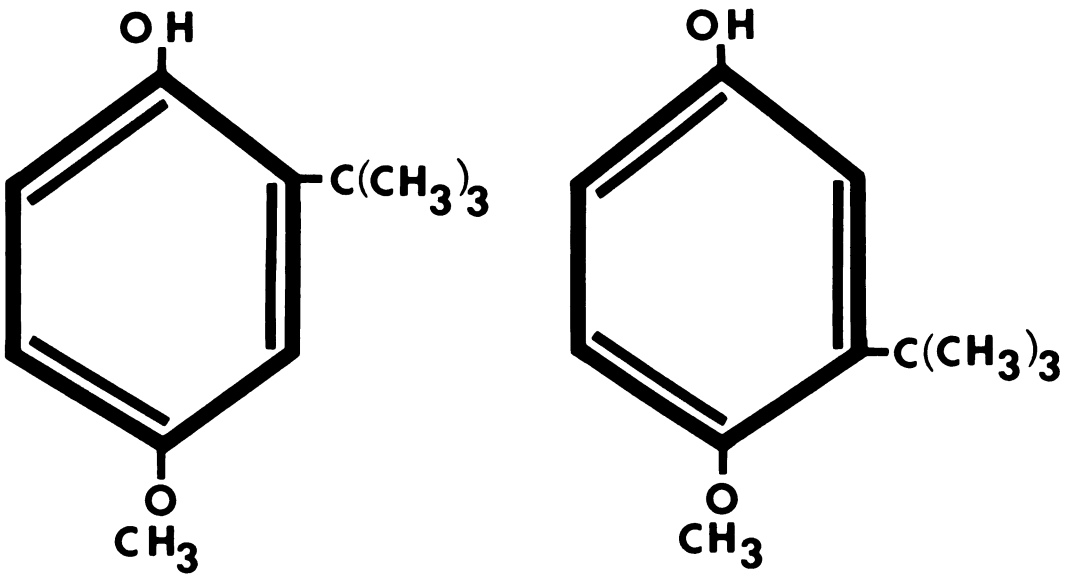
As of September 1978, the opinion of the Committee regarding BHA and BHT has not been released publically. It is possible that these antioxidants which entered the food supply without rigorous toxicological evaluation will remain in use. Therefore, knowledge of their metabolism and physiological effects is necessary.

Chemical Properties

BHA and BHT are members of a larger group of synthetic phenolic antioxidants (12). BHA is a mixture of isomers of MW 180 (Figure 1); 4-15% of 2-tert.-butyl-4-methoxyphenol and 85-96% of the 3-isomer. The melting point ranges from 54-58° and the boiling point from 264-270°. BHT (3,5 di-tert.-butyl-4-hydroxytoluene) has a single structure with MW 220 (Figure 1). It has a melting point of 71° and a boiling point of 265° (12).

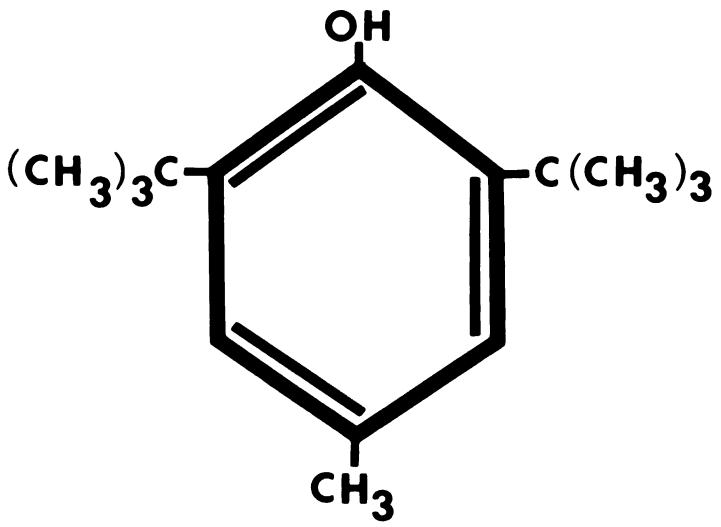
BHA and BHT are insoluble in water but soluble in alcohols, lipids, and various organic solvents. Both compounds have large hydrophobic binding abilities as measured by the degree of partition between liquid paraffin and Tris buffer at a physiological pH (13).

Figure 1. Structures of butylated hydroxyanisole (3-tert.-butyl-4-methoxyphenol and 2-tert.-butyl-4-methoxyphenol) and butylated hydroxytoluene (3,5 di-tert.-butyl-4-hydroxytoluene) (6).



BHA

Butylated hydroxyanisole



BHT

Butylated hydroxytoluene

Figure 1

Singer and Wan (14) found that BHT has significant effects on the physical characteristics of lecithin liposomes. Addition of 0.2 M BHT to the incubation medium reduced ^{22}Na efflux from these vesicles. BHT also increased the motility of fatty acyl side chains of the lecithin and reduced the temperature at which the chains "melt". These effects of BHT on the liposomes appear to be related to its structure, since similar studies with BHT analogs indicate that the methyl and hydroxyl groups give increasing degrees of effectiveness in preventing ^{22}Na efflux. BHT has also been found to decrease destructive oxidation of lecithin micelles (15).

Antioxidant Properties

The first event during the development of rancidity in fats and oils is the slow uptake of oxygen; this is called the induction period (3). Subsequently oxygen uptake is rapid, and rancidity soon becomes apparent.

This oxidation of fats involves free radical chain reactions (3,12). In the presence of light an oxygen molecule adds to a carbon-carbon double bond forming a peroxide radical. This radical may react at another double bond to form an unstable hydroperoxide and a free radical. These products may react further, with the initiation of a series of chain reactions. The final products are short chain volatile aldehydes, acids, and ketones which produce the characteristic flavor and odor of rancid fat (3).

The phenolic antioxidants act by donating hydrogens to the free radicals, thus ending the chain reactions. Small quantities of these antioxidants are needed because of the long chain lengths (12).

Intestinal Absorption

Because BHA and BHT are lipid-soluble compounds, it is probable that they would be well absorbed at the intestinal mucosa. The observation that BHT readily enters phospholipid vesicles *in vitro* (12) suggests that these compounds are transported *in vivo* in association with cholesterol-triglyceride micelles. Data supporting this postulation are lacking, however, and current estimates of absorption are based on indirect evidence from studies of excretion and distribution.

Daniel and Gage (16) studied the absorption and excretion of BHT by rats. Four days following an oral dose of ^{14}C-BHT (100 mg dissolved in olive oil), 24-37% of the label was excreted in the urine and 35-42% in the feces, a total of 59-79%. In another study, an intraperitoneal injection of 100 µg ^{14}C-BHT in ethanol was administered to rats (17). After four days, 32% of the label was excreted in the urine and 37% in the feces. This agrees closely with the values obtained by Daniel and Gage (16) and suggests that a large portion of the BHT derivatives found in the feces is due to biliary secretion (12,17), not failure of absorption. Based on the above data for urinary and biliary excretion, Hathway suggests a minimum absorption of approximately 68% (12).

Gor'kov *et al.* (18) determined the kinetics of absorption and elimination of BHT following oral administration to human oncological patients. A curve of BHT concentrations in the blood was obtained after a dose of 3 gm in powder form. A peak BHT concentration of 0.8-1.0 mg/l was noted at two hours following ingestion, but a 6 gm dose produced a concentration of only 0.9 mg/l. These results suggest that

a limit exists for absorption of the undissolved form of BHT. The total absorption from the dose of 3 gm was estimated to be 1-3%. The results of this study appear to conflict with the experiments which indicate that these compounds should be well absorbed. However, the authors note that BHT dissolved in corn oil was better absorbed in oncological patients than the powder (18). They suggested that the greater degree of absorption observed when BHT was dissolved in an oil vehicle may be due to either increased dissolution of the BHT or to more micelles available for transportation of BHT across the intestinal lumen.

Metabolism

BHA and BHT are metabolized by enzymes located in the liver; the possibility of extrahepatic sites of metabolism has not been examined. Probable metabolic pathways have been constructed based on the patterns of metabolites excreted by various species (6,12). In rats the 2-isomer of BHA may be conjugated at the hydroxyl or methyl substituent to form the glucuronide or sulfate derivatives (Figure 2). The 3-isomer, which represents about 96% of the compound, is conjugated solely with glucuronic acid at the hydroxyl group in most species (12). The major metabolite of BHA in humans also appears to be the glucuronide derivative, indicating similar pathways of metabolism between humans and rats. Dogs, however, may excrete up to 25% of a dose of BHA as the sulfate; this suggests that the 3-isomer may be conjugated as the sulfate in some species (12).

Because of the steric hinderance of the hydroxyl groups afforded by the t-butyl substituents, the oxidation of BHT occurs mainly at the methyl group in the 4 position (19). The major pathway in rats

Figure 2. Hepatic metabolic pathways for 2-tert.-butyl-4-methoxyphenol and 3-tert.-butyl-4-methoxyphenol (12).

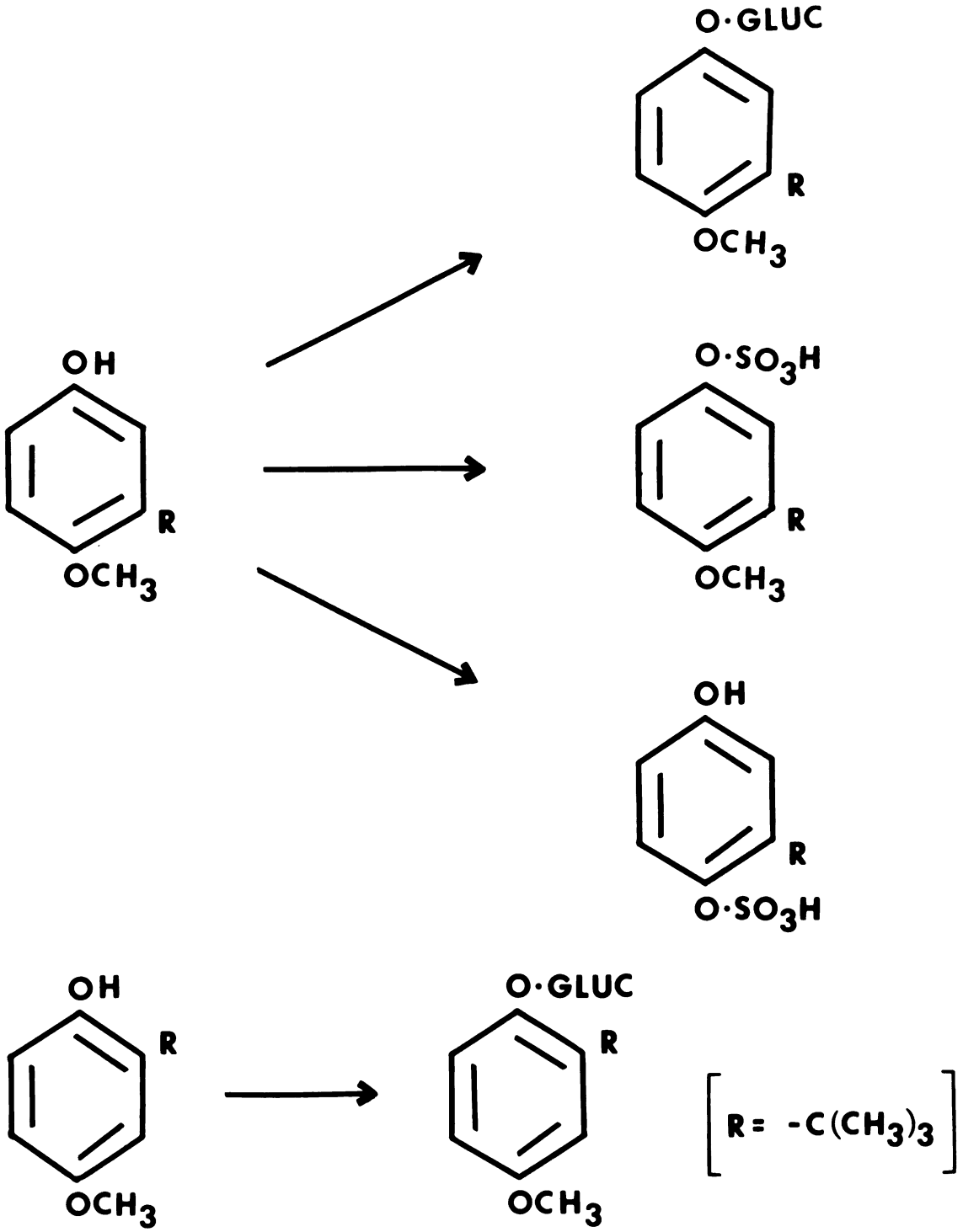

O·GLUC
R
OCH3
OH
R
OCH3
O·SO3H
R
OCH3
OH
R
O·SO3H
OH
R
OCH3
O·GLUC
R
OCH3
[R= -C(CH3)3]

Figure 2

(Figure 3) involves conversion of BHT to 2,6-di-tert.-butyl-4-hydroxymethylphenol (BHT-alcohol), with subsequent oxidation to 3,5-di-tert.-butyl-4-hydroxybenzoic acid (BHT-acid) and its glucuronic acid conjugates (20,21). The metabolites found in the greatest quantities in rat urine are BHT-acid, both free and conjugated, and a mercapturic acid derivative (20). In man, however, after a 5 mg/kg dose of ^{14}C-BHT, BHT-acid and its glucuronides were found in only minor quantities; the major metabolite was one where all 3 alkyl substituents had been oxidized to produce BHT-dicarboxylic acid (Figure 3).

The initial reaction from BHT to BHT-alcohol appears to be the rate-limiting step in elimination of BHT from the body. This may be inferred from studies with rats demonstrating that the excretion of BHT-alcohol is faster than that of BHT (21). Unlike BHT, no unchanged BHT-alcohol was found in the bodies of treated animals after 11 days (21).

BHT oxidase is the enzyme which catalyzes the initial step of conversion of BHT to BHT-alcohol and was first described in 1965 by Gilbert and Golberg (22). The major portion of the activity of this enzyme in rat liver homogenates was found in the microsomal fraction. Optimal activity was obtained under aerobic conditions at pH 7.4 and in the presence of NADP and a NADPH-generating system (glucose-6-phosphate and glucose-6-phosphate dehydrogenase). The enzyme was found to be competitively inhibited by SKF-525A. These properties indicate that BHT oxidase is a typical hepatic mixed function oxidase (MFO) (22).

The activity of BHT oxidase in untreated rats was found to be greater in males than females. When body weight was used as an index

Figure 3. Hepatic pathways of metabolism for 3,5 di-tert.-butyl-4-methoxyphenol (12).

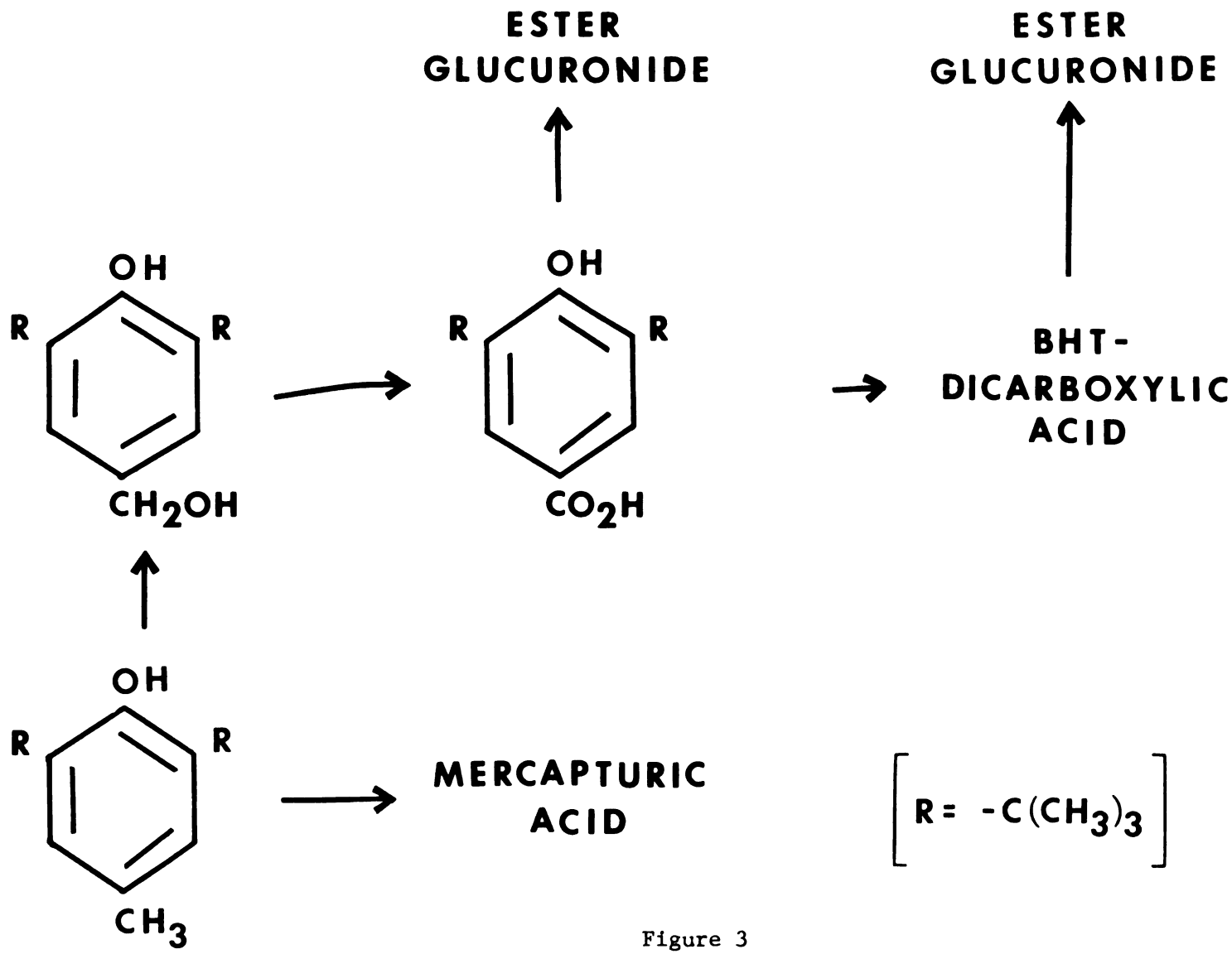

ESTER GLUCURONIDE
ESTER GLUCURONIDE
OH
R
R
CH2OH
OH
R
R
CO2H
BHT-DICARBOXYLIC ACID
OH
R
R
CH3
MERCAPTURIC ACID
[R= -C(CH3)3]

Figure 3

of age, the activity of the enzyme increased up to 275 gm, declining thereafter (22). The specific activity of the enzyme was increased as early as 24 hours following a single dose of 500 mg/kg BHT, and greater increases in activity were obtained with continued administration.

Distribution and Storage

Many of the characteristics of the physiological interactions of BHA and BHT are determined by their lipid solubility. After a dose of radioactive antioxidant, most of the label will be associated with lipid-rich tissue. Twenty-four hours after one dose of 44 mg/kg ^{14}C-BHT to male rats the label was distributed (in descending concentration) in the liver, fat, adrenals, skin, lungs, kidneys, and blood (23). In females the order was slightly different: fat, liver, skin, adrenals, lungs, and gonads. Female rats were found to have greater storage capacity than males for BHT: after three weeks of 0.5% BHT in the diet the levels in their fat reached a maximum of 66 ppm, compared to 45 ppm for males (16).

With repeated administration of BHT there was accumulation in body tissues: for males, the concentration in fat increased from 0.023% of the dose per gram of tissue following one dose to 0.065% following four; and in females, from 0.161 to 0.243% dose/gm (23). When rats were administered five doses of ^{14}C-BHT (44 mg/kg/day) and killed eight days after the last dose, the concentrations in the tissues of males fell with a half-life of 7-10 days(16). This decline was more rapid in fat, liver, and gonads than adrenals and blood. The concentrations in female tissues declined at approximately the same rates as those of

males, but remained at higher levels, especially in fat, blood, and gonads.

The accumulation of BHT in the body appears to be related to the activity of drug-metabolizing enzymes. Inasmuch as the rate of BHT metabolism was found to be slower in females (22), the concentrations in body tissues should be higher. In the first few days following administration of 500 mg/kg/day to rats, the BHT content of fat rose sharply, reaching 230 ppm after 2 doses in females and 170 ppm in males (24). Despite continued administration the concentration declined to a steady level of 100 ppm in both sexes within four days. This reduction appeared to be concomitant with increases in hepatic mixed function oxidase activities which attained maximal levels on days 3 and 4 of treatment. Female rats received various doses of BHT (10 to 500 mg/kg/day) for 7 days and the BHT concentration in adipose tissue measured colorimetrically (24). At low doses the slope of the dose-response curve was steep; but above 75 mg/kg/day--the threshold for drug-metabolizing enzyme stimulation--the concentrations increased much more slowly.

Excretion

After ingestion, the phenolic antioxidants are found in the feces of laboratory animals as the result of non-absorption, or biliary secretion of metabolites; and in the urine as water-soluble derivatives. BHA metabolites are excreted mainly in the urine. Within five days following a single dose of 400 mg/kg BHA (85% of the 3-isomer and 15% 2-isomer) dissolved in corn oil and administered by gavage, 60-80% of the dose was found in the urine of rats as glucuronides, 11-16% as

ethereal sulfates, and 2-7% was unchanged (25). Twenty-four hours after the administration of 1 gm BHA to rabbit does (1.6-2.3 kg body weight) approximately half of the dose was excreted in the urine as glucuronides, 9% as ethereal sulfates, and 6% as free phenols (26).

Within 24 hours of a dose of 1-2 mg ^{14}C-BHT to male rats, 6.4% was found in the urine and 5.2% in the feces (16). After seven days the cumulative totals were 24% in the urine and 66% in feces, or 90% in total. In female rats a greater percent (18%) was collected in urine after 24 hours while 7.9% was found in the feces. At the end of seven days, 37% of the label had been recovered in the urine and 72% in the feces, 109% in total. Pretreatment with 0.5% BHT in the diet for 21 days increased the percentage of a test dose excreted in the urine of male rats: 25% in 24 hours and 41% in four days(16). Dacre (27) found that four days following a single dose of 0.8 gm BHT to rabbits 54% could be accounted for in the urine; 36% as the glucuronide, 8% as ethereal sulfates, and the remainder as BHT-acid derivatives.

Daniel et al. (28) utilized ^{14}C-BHT to study the urinary excretion of BHT and BHT by humans. Approximately 50% of a dose of 40 mg BHT was excreted within 24 hours, and 50-60% of a dose of BHA. After 11 days, 63-67% of the BHT was excreted in the urine as well as 80-87% of BHA. BHT appears to be eliminated to a greater extent in the urine of human males, compared to rats.

The greater fecal excretion of BHT by rats may be due to a larger degree of biliary excretion. Four days following administration of labelled BHT to male rats the bulk of radioactivity in the carcasses was found in the gut (16). After a parenteral dose of

100 μg labelled BHT to male rats, 32% was excreted in the urine and 37% in the feces during 4 days (17); again, most of the radioactivity of the carcass was found in the intestines. Six hours following a similar parenteral dose, 52% appeared in the bile; and when this amount was given intravenously, 94% was secreted into the bile (16). A greater fraction of BHT metabolites was found in the urine of female rats, compared to males, and the overall rate of excretion was faster. This may be due to the more pronounced enterohepatic circulation of males. Collection of bile for forty hours following a dose of ^{14}C-BHT (1.75 mg) indicated that 53% of the amount administered was excreted via this route in males, and 17% in females (16).

Effects on Drug-Metabolizing Enzymes

Treatment with BHA or BHT has been shown to increase the activities of drug-metabolizing enzymes in the liver. Administration of 500 mg/kg/day BHA by oral intubation for 21 days led to transient increases of biphenyl-4-hydroxylase and BHT oxidase activities in rat livers, with a maximum on the second day (29). Rats treated with 330 mg/kg/day BHA for 6 days had no detectable changes in hexobarbital oxidase (HO) or aminopyrine demethylase (APDM) (13). Treatment with BHA at 500 mg/kg/day for 84 days had no significant effect on HO, APDM, and nitroanisole demethylase (NADM) activities in pregnant rats (24). However, because the enzymes were assayed only once in both of these studies, it cannot be ascertained that BHA did not affect their activities.

Administration of 50 mg/kg BHT by gavage to female rats twice daily for 10 days resulted in increased enzyme activities: aminopyrine metabolism was induced to 164% of control, hexobarbital metabolism by 154%, and butynamine, 354% (30). A larger dose of 150 mg/kg twice daily resulted in greater increases of 230%, 260%, and 495%, respectively. In vivo drug-metabolism was also tested, and pretreatment of animals with 50 mg/kg BHT twice daily for 5 days decreased by 60% the narcosis and ataxia of sodium pentobarbital (30 mg/kg). Addition of 0.01% BHT to the diets of female rats for 28 days had no detectable effects on APDM activity; but after 10 days of 0.1%, the activity was twice that of controls while a seven-fold increase was obtained with 1.0% in the ration (31). The activities returned to control levels 4-7 days following cessation of treatment.

The time course of the effects of BHT treatment on drug-metabolizing enzymes was established by Gilbert and Golberg (24). The activity of HO was increased after one dose of 500 mg/kg BHT, and NADM and APDM were induced following two days of treatment. After four days the enzyme levels reached a plateau and remained at that level for the 21 days of the experiment. A dose-response curve for the effects of BHT treatment on the activities of HO and APDM was also constructed. Groups of female rats were given graded doses of BHT between 10 and 500 mg/kg/day by gavage for seven days. The threshold for induction of activities of both enzymes was between 25 and 75 mg/kg, with a linear increase in activity as the dose was increased. Gaunt et al. (32) studied the effects of dietary restriction on BHT-induced enzymes. They concluded that restriction of food to half the ad libitum intakes did not affect the response to BHT of HO, NADM, or APDM.

In contrast to the studies with rats, treatment of juvenile monkeys with BHA produced greater increases in enzyme activity than did administration of BHT (33). After treatment with 500 mg/kg/day of BHA, the activity of NADM was increased 45% at the end of two weeks, and 83% by four weeks. The effect of 500 mg/kg/day BHT was not as pronounced, resulting in 28% increases in activity after two weeks of treatment and 67% after four. The level of cytochrome P_{450} was not altered by treatment with either antioxidant in this study.

Effects on Organ Weights

Many compounds which induce hepatic drug-metabolizing enzymes concomitantly increase the liver weight of treated animals (13,34). A 25-30% increase in the liver weight/body weight (relative liver weight) of rats was observed following two doses (500 mg/kg/day) of either antioxidant (13). Despite continued administration, the relative liver weight (RLW) of BHA-treated animals declined to control values by day 6 of treatment; those of BHT-treated rats were further elevated to 35-40% by the eighth day (35,36). After a 14 day recovery period the liver weights of treated rats were not significantly different from those of controls (37). There were no apparent effects of BHA on the liver weight of dogs when up to 100 mg/kg of the antioxidant was incorporated into the diet for 1 year (38). Again, because of the transient nature of the increase in RLW produced by BHA treatment, it is not possible to conclude that an increase did not occur during that time.

Incorporation of 0.1-0.4% BHA into the ration of rats for six weeks resulted in elevations in RLW which varied directly with the

concentration (39). The liver weight of BHT-treated animals, in contrast, attained a plateau at 0.2% of the diet. At all levels of antioxidant in the diet the liver weight of animals receiving BHT were greater than those of BHA-treated rats (39,40). In contrast to the studies with rats, BHA treatment (500 mg/kg/day) for four weeks had a greater effect on the RLW of juvenile monkeys than a comparable regimen with BHT (33).

The relationship between restricted feeding and alterations in liver weight was examined in animals treated with 500 mg/kg/day BHT for 14 days (32). Restriction of food to half the ad libitum intakes resulted in lighter livers compared to fully-fed/non-treated rats, but the RLW were comparable between the fully-fed/treated and deprived/treated groups. Food deprivation did not adversely affect the liver weights during a 14 day recovery period. Similarly, the response to addition of 0.05-0.5% BHT to the ration is not affected by decreasing the protein level of the diet to 4% (41).

Treatment with BHA or BHT also appears to affect the weight and ascorbic acid content of the adrenal glands. A 6.7% increase in adrenal weight was observed 48 hours following a dose of 500 mg/kg BHT to rats (42) while the ascorbic acid content increased 4%. In an 8 week study (43), rats receiving 0.2% BHT in the diet had adrenals which were 25% heavier than the untreated animals, and the ascorbic acid content was increased 39%. The authors interpreted these results as indicative of stress from the BHT treatment. Gaunt et al. (36) observed that the relative adrenal weights of female rats were elevated after ingesting a ration containing 0.1% BHA or BHT for 12 weeks.

These investigators suggested that rather than an effect of stress, the results indicate hyperfunctional enlargement due to the presence of drug-metabolizing enzymes in the adrenals.

Saheb and Saheb (44) found that the relative brain, kidney, and heart weights of rats were increased as a result of incorporation of 0.5% BHT into the diet for seven weeks. This is likely due to the reduction of body weight gain which occurred in treated animals. In another study (40) however, no effects were observed in the relative weights of the spleen, heart, or kidneys when animals were fed a similar ration for up to two years.

Histological and Biochemical Changes

Many investigators have characterized the histological and biochemical changes which occur during the period of liver growth induced by BHA and BHT. Astill and Mulligan (45) administered 225 mg/kg of BHA or BHT by gavage and examined the livers microscopically. Forty-eight hours later, hepatic abnormalities were noted in 30% of the animals receiving BHT and in 50% of those treated with BHA. These changes consisted of alterations in color, rounded margins, and small yellow foci. In the livers of juvenile monkeys given 500 mg/kg/day of BHA or BHT by gavage for 4 weeks, proliferation of the smooth endoplasmic reticulum (sER) was observed, as well as an increase in lipid droplets (33). A lesser degree of change was found following BHT administration. Approximately 15% of the cells from animals treated with either antioxidant had fragmented nucleoli; and with BHA treatment, long fibrils were randomly dispersed throughout the nucleolus.

No changes occurred in hepatic protein, DNA, or RNA contents of the livers. Increases were observed in the SER of livers from rats fed diets containing 1.0-5.0% BHT for up to 28 days (31). Maximal effects were noted two days after cessation of treatment (day 30), although by days 36-37 the amount of sER had decreased to control levels. Microscopic examination revealed the presence of fatty vacuoles, but no mitochondrial damage was apparent.

Incorporation of 0.1-0.5% BHT into the diet of male rats for 96 days led to a slight increase in the hepatic water concentration (2%), while liver weight increased 13% (46). The content of neutral fat was depressed 2%; the protein concentration did not change. The concentration of hepatic glycogen increased from 1.7% in control animals to 3.4% in those receiving 0.1% BHT, and to 6.6% for the rats ingesting a diet with 0.5%. In these experiments, the concentration and content of DNA in the liver also declined and the RNA/DNA was elevated. Following a dosage regimen with BHT which resulted in 29% increases in RLW (30 mg/kg/day x 10 days) the concentration of protein was reduced by 12%; however, the total content was raised 14% (47). The concentration of DNA in these livers was depressed 7% and RNA by 5%. The DNA and RNA contents were elevated by 17% and 23%, respectively. The ratio of RNA to DNA increased 8%, although the protein/DNA did not change.

Kerr et al. (48) administered BHT in the diet of rats for 24 days at 0.8-2.8 mg/cal (approximately 50-150 mg/kg/day), with pair-fed control animals. A 60% increase in liver wet and dry weights occurred. The total protein in the liver was elevated 60% and the incorporation of ^{14}C-leucine into hepatic protein also increased by the same amount. There were no changes in glycogen or total lipid contents. No

abnormalities of liver cytology were observed, though a 20% increase in the cytoplasm to nucleus ratio occurred. Following the first four days of BHT treatment there was a ten-fold increase in the incorporation of tritiated thymidine into DNA, as well as an increase in the mitotic index. Both of these parameters returned to control levels despite continued administration.

Lane and Leiber (49) followed the same dosage protocol as that of Kerr. All animals receiving BHT in the diet had augmentation of sER in some hepatocytes: after 1-2 days increases were noted in 20-50% of the cells, and after 3-7 days the incidence was 70-80%. No changes were found in the nucleolus. In the first four days of treatment there was increased mitotic activity in two-thirds of the samples. In contrast, after a single dose of 1.4 gm/kg body weight, there was no increase in mitotic activity evidence at any time.

Acute Toxicity

The median lethal dose of a compound (LD_{50}) is often used as a comparative index of toxicity. The oral LD_{50} of BHA for mice is 2,000 mg/kg body weight, and for rats it is somewhat higher, ranging from 2,200-5,000 mg/kg (11). The LD_{50} of BHT is 1,700 mg/kg for male rats and 1,970 mg/kg for females (8). In larger animals the approximate lethal dose of BHT is 940-2100 mg/kg for cats and 2100-3200 mg/kg for rabbits (8).

Prior to death the fatal doses of BHT resulted in complete loss of appetite, increased salivation , miosis, increased urination, diarrhea, tremors, and paralysis of hindquarters (8). Eventually

the animals developed respiratory difficulties and coma. Intravenous infusion of 40 mg/kg BHT to dogs produced an immediate drop in blood pressure, from 95 mmHg to 32 mmHg (8). In consideration of the fact that atropine sulfate afforded a slight degree of protection against this--as well as the previously mentioned symptoms--the authors suggested that the toxic effects were partially mediated through stimulation of the parasympathetic nervous system.

The responses of laboratory animals to the phenolic antioxidants are modified by various genetic and environmental factors, such as:

a) Species and age. In the experiments of Day et al. (50) supplementation of the diets of rats with 10-20% lard depressed the concentrations of serum cholesterol in rats over the course of five weeks, although food intake rose by 20 gm during that period. This is contrary to the elevation of cholesterol which would be expected in humans; this may complicate extrapolation of results from experiments designed to test the effects of BHT on lipid metabolism. In monkeys BHA has more profound effects than does BHT (33) although the reverse is true in rats, the species most used for toxicological studies. In addition, these authors demonstrated that the decreased enzyme activity (glucose-6-phosphatase) noted with BHA administration to juvenile monkeys was not apparent in infants.

b) Sex. Many investigators have established a sex difference in the effects of phenolic antioxidants. Brown et al. (40) demonstrated that BHA or BHT addition at 0.5% of the diet produced greater depression of growth in male rats. In contrast, BHT levels of 0.01-0.1% in the diets fostered weight gain in male

rats, but not females (44). The relative liver weight of male rats was increased when 0.1-0.5% BHT was included in the diet. In rats treated with 500 mg/kg BHT for one week, G-6-Pase levels remained depressed 2 weeks after cessation of treatment in females only.

c) Diet. It has been observed that the toxicity of some xenobiotics may be modified by dietary manipulation (51); this also appears to be true for BHT. At a level of 24% protein for 28 days, the acute LD_{50} of BHT was 3900 mg/kg, with 8% protein it was 2150, and with 4%, 1350 mg/kg. The toxicity of BHT was potentiated by increasing the percentage of lard in the diet as demonstrated by decreased growth (40). The dosage level at which liver lipids of BHT-treated animals were elevated relative to controls was decreased when food was restricted to one-half of ad libitum intakes (43). With restricted feeding, BHT treatment resulted in greater plasma cholesterol, phospholipids, and triglycerides (44).

Such factors may contribute to difficulties in comparing the results of various studies, and a lack of standardization in the toxicological literature concerning BHA and BHT has made interpretation of conflicting data difficult.

Effects on Growth

The incorporation of 0.1% BHA or BHT into the diets of weanling rats for 16 weeks did not affect food intake or growth rate, except at 16 weeks for the males receiving BHA (36). A depression of growth occurred when the food consumption per unit body weight was greater

in the treated animals, suggesting a reduction of the efficiency of food utilization. Pascal et al. (46) observed that BHT adversely affected the weight gain and food consumption of weanling rats when incorporated for 10 weeks into a ration containing 13% protein. The amount of food ingested per gram of weight gain was 3.54 in control animals, 3.70 with 0.1% BHT, and 4.71 for 0.5% BHT. Treatment with BHA at concentrations of up to 0.5% of the ration for six weeks had no effect on the body weight of rats; but 0.3% BHT depressed the weight gain of males, and to a lesser degree, of females. Gaunt et al. (32) found no effect of BHT treatment (500 mg/kg/day x 14 days) on the growth of rats. Takanaka et al. (30) administered 150 mg/kg BHT twice daily to female rats for 10 days. During this time the weight gain of the BHT-treated animals was almost triple that of the control animals. Rats fed a 20% casein diet for eight weeks with the incorporation of 0.2% BHT had greater food intake and growth than untreated rats, as well as increased protein efficiency (43).

Effects on Enzymes

Schöebesch (42) detected an increase in catalase activity in the blood of male rats receiving 250-500 mg/kg BHT, but no effect was observed on the number of red blood cells, the hemoglobin content, or mean corpuscular volume. The increase in activity was not apparent when BHT was fed in the diet at 0.2% (43). The addition of 250-600 mg/kg BHA to the ration of rats depressed plasma catalase and peroxidase activities (52). Administration of 500 mg/kg BHA to rats for 21 days resulted in increased activity of hepatic UDPG-dehydrogenase after the second day of treatment; this remained elevated for the duration of

the experiment (29). Karplyuk (53) measured serum protein, catalase, and peroxidase in rats treated with 8 mg/kg/day BHA or BHT for six months; no changes were observed in these parameters, or in coagulation. In another study (54) the activities of blood amylase and pancreatic lipase were in the normal range, as well as those of alkaline phosphatase and enterokinase of the small intestine. Administration of BHA or BHT at 225 mg/kg by gavage had no effect on serum transaminases (45). Treatment with 300 mg/kg/day for 10 days, or at 0.3-0.5% of the diet for 87 days, depressed the free form of acid phosphatase in the liver of rats, but not the reserve form packaged inside the lysosomes (55). The authors postulate that this was due to stabilization of the lysosomal membrane rather than a direct effect on the enzyme.

Lipid Metabolism

Several authors have observed increases in hepatic fatty vacuoles in association with BHA or BHT administration to laboratory animals (31,33). After 3 weeks of treatment with 500 mg/kg/day BHT, the serum cholesterol level of monkeys was depressed; this, however, returned to normal by 4 weeks (33). There was an increase in total lipids in the livers of monkeys treated with corn oil alone (11.2%) relative to non-treated animals (5.9%). The lipids in the livers of monkeys treated with BHA (7.3%) and BHT (12.5%) were not significantly different from the corn oil group. The percentage of rats showing "fatty changes" in the liver was increased after administration of 50-500 mg/kg BHT for one week (37), although to a lesser extent than ethionine or carbon tetrachloride. In this study no abnormalities were noted with the same treatment schedule of BHA.

Incorporation of 4 mg/kg/day BHA or BHT into a ration containing 25% lard did not adversely affect body weight or food intake during the 35 days of the experiment (56). No changes were observed in the ketone body concentration, cholesterol, or phospholipids in the serum of treated animals. The cholesterol level was elevated in the serum of rats consuming greater than 0.1% BHA in the diet for six weeks: BHT had a larger effect which was proportional to the dose (39). No changes in the concentration of cholesterol or polyunsaturated fatty acids occurred in the livers of rats treated with BHT. Sporn and Schöbesch (43) fed rats a diet with 20% casein and 12% pork fat and found that addition of 0.2% BHT slightly increased food intake over the course of 8 weeks. The total lipid content of the livers of treated animals was elevated; this effect was enhanced with a low protein diet devoid of choline. Gaunt et al. (32) observed that BHT treatment (500 mg/kg/day x 2 weeks) increased the levels of serum cholesterol and phospholipids regardless of food intake; these returned to control values after a recovery period of 14 days.

Reproduction

Brown et al. (49) studied the effects of BHA or BHT incorporated at 0.01-0.5% into a diet containing 10-20% lard. There were no effects on reproduction--number of pups born and weaned, or weight of the litters--from dams fed diets incorporating up to 0.5% of either antioxidant since weaning. A low incidence of anopthalmia was found in the offspring when the parents had been fed BHT for five months; none was found in the control or BHA-treated animals.

Karplyuk (53) conducted a three generation study of rats and dogs receiving the phenolic antioxidants BHA, BHT, or propyl gallate at 0.2% of the diet for 6 months, then 0.4% for another 6 months. Both the F_1 and F_2 generations subsequently received 8 mg/kg. No morphological or biochemical abnormalities were found in any organ system, nor was any effect found on reproduction. Addition of 0.125-1.55% BHT to the diets of pregnant rats on day 1 of gestation indicated that only at the highest concentration was there significant fetal death or weight loss in the dams (57). Clegg (58) studied the effects of BHA or BHT by administering one large dose of 1000 mg/kg on a specific day of pregnancy, or smaller doses (250-750 mg/kg/day) for several days: the offspring were not affected by either compound.

There is evidence that BHT may be unidirectionally transported across the placenta. In a study examining the transplacental accumulation of volatile organic compounds using mass spectroscopy/gas liquid chromatography, BHT was found in the cord blood of an infant whose mother had none in her blood (59). Following administration of 500 ppm of the diet to hens, 20 ppm were found in egg lipids; however, this did not appear to harm the embryo (12).

Influence on Carcinogenesis

From the information available on the metabolism and chemical properties of BHA and BHT, it is possible that either compound may alter the carcinogenicity of various substances by:

a) Decreasing free radical initiation through its antioxidant properties.

b) Altering nucleic acid metabolism.

c) Increasing hepatic xenobiotic metabolism. This may increase the formation of carcinogens from pre-carcinogens, or conversely may shunt the carcinogenic compound through pathways producing more inocuous metabolites.

BHT has been tested in the U.S.S.R. as a therapeutic agent. In clinical studies it has been shown to be effective for the treatment of tumors of the urinary bladder and for trophic conditions of the skin (18).

Single injections of 70, 115, or 200 mg/kg BHT 7 days following the introduction of Ehrlich's ascites tumor cells into mice depressed the incorporation of ^{14}C-amino acids into proteins of the ascites cells and ^{14}C-adenine into nucleic acids (60). No effect of BHT was seen in the livers and kidneys of mice not receiving the tumor cells. The authors postulated that the rapidly dividing cells are more ssceptible to the actions of phenolic antioxidants.

Pretreatment of rats with BHT (0.5% of the diet for 7 days) prior to injection of labelled 2-acetylaminofluorene (AAF) was found to decrease the number of AAF-DNA adducts in liver cells by 50% (61). The growth retardation caused by addition of 0.05% AAF to the diet was reduced 50% by the simultaneous inclusion of 0.5% BHT. Peraino et al. (62) fed rats a ration containing 0.02% AAF for 18 days, and returned them to a control diet for seven days. For the next 200-407 days the animals were fed either a control diet or a diet containing 0.05% phenobarbital or 0.5% BHT (200-500 mg/kg/day). Compared to the phenobarbital-treated groups, animals fed BHT had fewer hepatic tumors, although the incidence was still higher when compared to animals fed the control diet.

Following the injection of leukemia cells to rats the levels of free radicals increase to a maximum at 4 days, even before other evidence of the leukemic process is apparent (63). Administration of 150 mg/kg/day BHT after inoculation with such cells prevented the increase in radicals, as well as elevation of spleen weight. When administered before and during treatment with azoxymethane, BHT (6600 ppm) was found to reduce the incidence of intestinal tumors induced by that agent (64). However, when administered before azoxymethane, there was a slight increase in the number of tumors. This phenomenon was also noted following a similar schedule with phenobarbital.

Effects on Other Physiological Systems

Addition of 2 mg/ml BHA to the perfusion medium of an *in situ* intestinal preparation was found to decrease the absorption of glucose and methionine, and increase water absorption (65). Histological changes and edema were noted in the bathed tissue. Posati *et al.* (66) observed that BHA attenuated the constriction of smooth muscle noted with bradykinin, apparently by a competitive action. The threshold at which BHA depressed the contractile response of guinea pig ileum was 8×10^{-9}M. Donaldson (67) suggested that BHA reacts directly with bradykinin inasmuch as preincubation of muscle with BHA did not have the same inhibitory effect as preincubation of the bradykinin medium with BHA.

In a series of articles examining the pathology of chemically induced lung damage using BHT as a model toxin, Witschi *et al.* (68,69, 70) have delineated the effects of BHT on this organ. Intraperitoneal injections of 400 mg/kg BHT into mice resulted in increased lung

weight, edema, and proliferation and enlargement of interstitial cells. Electron microscopy revealed increases in lamellar bodies of the cells, and enlargement of nuclei and nucleoli. The contents of DNA and RNA in the lungs also increased, as well as the incorporation of ^{3}H-thymidine into DNA. These biochemical changes are believed to be secondary to cellular necrosis, and represent reparative phenomenon rather than a direct effect on DNA and RNA syntheses.

Effects on the Kidneys

Although histological changes have not been observed in the kidneys from animals treated with either antioxidant, several authors have reported impairment of urinary concentrating ability in association with administration of BHA or BHT. Deichman (8) noted increased urination by various laboratory animals receiving fatal doses of BHT. Denz and Llaurado (9) examined the effects of five doses of BHA or BHT (approximately 500 mg/kg/day) on the sodium and potassium balance of rabbits. The daily urinary sodium and potassium excretions were found to increase markedly despite a reduction of food intake. The levels of serum sodium remained constant; however, the potassium concentration declined to half the control values by the sixth dose. An increase in urinary aldosterone excretion was noted: the authors postulated that this represented hypersecretion in compensation for an inability of the nephron to reabsorb sodium. It should be noted that these animals were afflicted with an inflammatory disease of the kidneys prior to inclusion in the study.

Little information is available regarding the effects of BHA, BHT, and other food additives on renal function although the kidneys perform many functions which are sensitive to biochemical and physical perturbations. Adverse effects of foreign compounds could be manifest as increased excretion of glucose, protein, or renal enzymes, alterations in the ability of the kidneys to concentrate urine, changes in transport processes, and morphological abnormalities (71). No single test is sufficient to detect the myriad of possible effects that a substance may exert on renal function. The use of a few selective techniques, however, may provide information on renal integrity; more powerful methods may then be employed if indicated.

A number of transport systems for the secretion of endogenous and exogenous organic ions exists in the proximal tubule of the nephron; these are dependent upon patent biochemical and structural machinery. During the process of secretion in the intact animal, organic acids and bases are actively accumulated from the blood by separate systems, across the peritubular membrane into cells of the proximal tubule. Movement into the lumen is then accomplished by passive diffusion down a concentration gradient which is maintained by constant tubular flow. In vivo, inhibition of the secretory apparatus for an ion is demonstrated by depression of the maximal transport capacity, T_m. However, interpretation of such data can be confounded by any changes in renal blood flow or humoral factors which accompany treatment (72).

In order to eliminate such variables, an in vitro method described by Cross and Taggart is used to estimate secretory capacity (73). Renal cortical slices are incubated in a medium containing a prototype

organic acid, para-aminohippurate (PAH), and a prototype base, N-methylnicotinamide (NMN) or tetraethylammonium (TEA). The ability of proximal tubular cells in the slices to accumulate these ions from the medium is expressed as a ratio of the concentration of the ion in the slices to the concentration in the medium (S/M). The magnitude of the gradient established is an index of transport across the peritubular membrane of the cells (74).

Although the normal morphology of the nephron is disrupted in the slice system, this method has been shown to have validity for the comparison of the effects of toxic compounds on secretory processes (74). Substances such as lactate and acetate--which stimulated the transport of PAH in vivo--also increased the PAH S/M in the slice system. Those compounds which depressed the T_m of PAH by competitive (probenecid, penicillin G) or other means (succinate, 2,4-dinitrophenol) did so in vitro. Because of its dissociation from hemodynamic and humoral alterations, the slice technique is at present the most sensitive method for detection of toxic effects on organic anion and cation transport (71).

Loss of the ability to concentrate urine may be the result of gross damage to tubular cells, poisoning of ionic pumps which foster water reabsorption, or loss of the medullary concentration gradient. As a result, copious amounts of dilute urine would be produced. Conversely, anuria or oliguria may be a consequence of reduced filtration, tubular blockage by cellular debris, or alterations of interstitial and luminal pressure (75). Therefore, measurement of urine volume and osmolality may also provide an index of renal integrity.

That BHA and BHT might be nephrotoxic was suggested by the studies which noted increased urination and inappropriate electrolyte excretion (8,9). These observations, however, were from morbibund animals. In addition, the effects of antioxidant treatment on renal tubular transport mechanisms have not been examined, although a large proportion of the anionic metabolites of these compounds are excreted in the urine.

In order to systematically examine the effects of BHA and BHT on the kidneys of healthy animals, a series of experiments was designed to: 1) determine whether treatment with either antioxidant affects renal secretory processes for organic ions; 2) quantitatively describe the effects of BHA and BHT treatments on renal concentrating ability, particularly with reference to intakes of electrolytes and water; and 3) examine the influences such factors as food intake and induction of drug-metabolizing enzymes might have on the results.

MATERIALS AND METHODS

Animals and Diet

Adult male Sprague-Dawley rats, 275-300 gm body weight, were purchased from Spartan Research Farms, Haslett, Michigan. The animals were individually housed in stainless steel metabolism cages and offered a grain diet free of antioxidants (Table 1) and distilled water, ad libitum. Prior to initiation of the experiments, the animals were allowed to adjust to the metabolism cages for 3-4 days. Body weight, water and food intakes, urine volume and pH, and urinary sodium, potassium, and osmolality were monitored daily for each rat, commencing one day before the start of the treatments.

In Vivo Treatments

BHA and BHT were dissolved in corn oil (10 mg/100 ml) by warming to 60°C in a water bath with gentle shaking. The prepared solutions were stored in amber bottles at room temperature. BHA or BHT was administered daily to rats by gavage at a dose of 500 mg/kg for 1, 2, 4, or 6 days. Control animals received an appropriate volume of corn oil. The animals were killed 24 hours following the last dose. At the time of death, the body weight and weight of liver, kidneys, and adrenal glands were recorded.

TABLE 1

Composition of Grain Diet

Ingredient	g/100 g diet
Ground Corn	60.7
Soybean meal	28.0
Alfalfa meal	2.0
Fish meal	2.5
Dried whey	2.5
Limestone	1.6
Dicalcium phosphate	1.75
Iodized salt	0.5
Minerals and Vitamins	0.45

(Ref. 76)

Organic Ion Accumulation

Animals were killed by cervical dislocation, and the kidneys quickly removed, decapsulated, and placed in cold 0.9% saline. Thin slices of the renal cortex were prepared freehand and divided between duplicate beakers containing 2.7 ml of the Cross and Taggart medium (73). The medium was adjusted to pH 7.4, and contained 7.4×10^{-5}M PAH (Eastman Kodak) and 6.0×10^{-6}M (2.5×10^{-2} μCi/ml) ^{14}C-NMN (New England Nuclear). The samples were incubated in a Dubnoff metabolic shaker at 100 rpm in an atmosphere of 100% oxygen at 25°.

After 90 minutes the slices were blotted, weighed, and homogenized in 5.0 ml of 10% trichloroacetic acid (TCA). A 2.0 ml aliquot of the incubation medium was added to 3.0 ml of 10% TCA. The volumes of the slice and medium preparations were brought to 10.0 ml with distilled water and the samples centrifuged at 1400 rpm for 10 minutes.

One ml aliquots of the slice and medium supernatants were assayed for PAH by a colorimetric method (77). NMN concentrations were determined in the supernatants with liquid scintillation counting. One ml aliquots were placed in glass vials and 10 ml of a modified Bray's solution (6 gm 2,5-diphenyloxazole and 100 gm napthalene per liter of dioxane) added. Each sample was counted for 20 minutes in a Beckman LS-250 liquid scintillation counter using an internal standard; the average counting efficiency was 50%.

The slice to medium ratios (S/M) of PAH and NMN were calculated as the concentration of the organic ion in the tissue, divided by the respective concentrations in the media.

Twenty-four Hour Urine Collections

Urine was collected into glass flasks containing toluene to prevent microbial contamination. At the end of 24 hours the pH and volume of each sample was recorded and 5 ml aliquots frozen in plastic test tubes. At a later date the samples were thawed, centrifuged, and sodium and potassium concentrations assayed by flame photometry (Instrumentation Laboratories, Inc., Model 143). The osmolality of the samples were determined by freezing point depression (Advanced Instruments Osmometer, Model 3L).

Enzyme Assays

Livers were removed and the weights recorded. For each animal, one lobe was weighed and homogenized in approximately 2.5 volumes of ice-cold 1.15% KCl by 4 passes of a Potter-type homogenizer. The 9,000 x g supernatant was prepared from each homogenate by centrifugation for 20 minutes at 5°. The supernatant was decanted and assayed for protein by the method of Lowry et al. (78).

BHT oxidase was determined by modification of the method of Gilbert and Golberg (22). The incubation medium contained 3.3 mM $MgCl_2$, 16.6 mM nicotinamide, 0.083 mM NADP, 2.5 mM glucose-6-phosphate (G-6-P), 0.06 IU/ml G-6-P dehydrogenase, 0.85 mM BHT, and 85 mM phosphate buffer. The reaction was initiated by adding 0.1 ml of the liver supernatant to beakers containing 3.0 ml of the incubation medium. The samples and tissue blanks were incubated in a Dubnoff metabolic shaker at 37°C under an atmosphere of 100% oxygen, at 120 rpm. The reactions were stopped at the end of 12 minutes by swirling each beaker and pouring the contents into a test tube containing 2 gm NaCl and 8 ml of

heptane-isoamyl alcohol. After extraction of metabolites as described by Gilbert and Golberg (22) the concentration of BHT-alcohol was determined using Gibb's reagent as the chromagen. The samples, blanks, and standard curves were read at 580 μm. The specific activity was expressed as nM of BHT-alcohol produced per hour per mg supernatant protein.

Hexobarbital hydroxylase activity was determined as a positive control using the method of Kupfer and Rosenfeld (79). The incubation medium contained 11.2 mM $MgCl_2$, 1.0 IU G-6-P dehydrogenase/ml, 12 mM G-6-P, 0.4 mM NADP, 85 mM phosphate buffer, and 2.68 μmole (8.4 μCi/μmole) of ^{14}C-hexobarbital (New England Nuclear). The reaction was started by adding 30 μl liver supernatant to beakers containing 1.0 ml of the medium, and terminated after 12 minutes with chilled citrate buffer. Incubation conditions were similar to those for BHT oxidase. The radioactive metabolites were isolated by several extractions as described by Kupfer and Rosenfeld, and assayed by liquid scintillation counting. The specific activity of the enzyme was expressed as DPM of radioactive metabolite isolated/hr/mg supernatant protein.

Effects of BHA and BHT on Organic Ion Transport In Vitro

In order to determine whether the unmetabolized forms of the antioxidants can inhibit PAH and NMN transport, BHA or BHT was added to the medium in which slices from untreated animals were incubated. Both antioxidants are hydrophobic, and since solubilizers such as dimethylsulfoxide have not proven satisfactory for use in the slice preparation, BHA or BHT was dissolved in absolute ethanol. One ml of the appropriate concentration of antioxidant, or 1.0 ml ethanol, was

added to a beaker and the ethanol allowed to evaporate, leaving the antioxidant as a film on the glass. The incubation medium was then added. If all of the antioxidant had gone into solution, the concentrations in the beakers would have been $1.5x10^{-4}$, $1.5x10^{-3}$, $1.5x10^{-2}$, or $1.5x10^{-1}$M. Actually a small, unknown fraction of the films did dissolve.

Cortical slices were prepared from the kidneys of untreated rats. The slices were pooled and divided between 10 beakers: duplicates of the control (ethanol only) and of 4 concentrations of BHA or BHT. The slices were incubated and organic ion transport determined as previously described. BHA, however, interfered with the spectrophotometric assay for PAH. In this case ^{14}C-PAH was used and NMN accumulation determined in separate beakers.

Induction of Drug-metabolizing Enzymes

Groups of rats received intraperitoneal injections of saline or 100 mg/kg phenobarbital for 3 days. These groups were then subdivided and half of each given four daily doses of 500 mg/kg BHT by gavage; the other half received an appropriate volume of corn oil. This experimental design employing two pretreatments and two treatments resulted in four groups: a saline/corn oil control group (S/C), a phenobarbital/corn oil group (P/C), saline/BHT (S/B), and a group that was pretreated with phenobarbital before treatment with BHT (P/B).

The animals were killed 24 hours following the last dose of BHT. Organic acid accumulation by renal cortical slices was determined, as

well as the specific activities of BHT oxidase and hexobarbital hydroxylase in the liver supernatants.

Statistical Analysis

Data from the *in vivo*, feeding, and drug-metabolism studies were subjected to a factorial analysis of variance, and that from the *in vitro* experiments to randomized complete block analysis. Differences between the means were tested by either Tukey's w-procedure or Duncan's multiple range test, using 0.05 as the criterion of significance (80).

RESULTS

In Vivo Study

Organic ion transport. Previous investigation had established that no difference exists in organic ion transport between sham-manipulated animals (PAH S/M 11.64±0.86, NMN S/M 6.92±0.37) and corn oil treated animals (PAH S/M 11.07±0.85, NMN S/M 6.52±0.17). Therefore, only the corn oil treated groups were used in these studies.

The ability of renal cortical slices to accumulate PAH was depressed in rats 24 hours following 1 dose of BHA (S/M 6.33±0.44) or BHT (6.11± 0.95) compared to animals treated with corn oil (8.94±0.76) (Figure 4). This depression was also evident in both treated groups after two doses of either antioxidant; the PAH S/M for BHA treated animals was 6.50± 0.47, for BHT 4.80±0.51, and for the control animals, 10.52±0.70. Following four doses the S/M for slices from BHA treated rats (8.08± 0.24) had begun to approach control values (10.19±1.13), while the S/M of the kidneys from BHT-treated animals reached a minimum (3.96±0.78). Despite continued administration of the antioxidants the organic acid transport capacity of slices from animals treated with BHA (7.48±1.02) or BHT (7.51±1.23) did not differ from control (8.64±0.70) after six doses.

There were no significant effects of either antioxidant on organic base transport as measured by NMN accumulation (Figure 5).

Figure 4. Accumulation of PAH by cortical slices from kidneys of rats treated with butylated hydroxyanisole or butylated hydroxytoluene. Each point represents the mean ± S.E.M. of four experiments. Asterisks indicate a statistical difference from the corresponding corn oil group at each dosage level ($p<.05$).

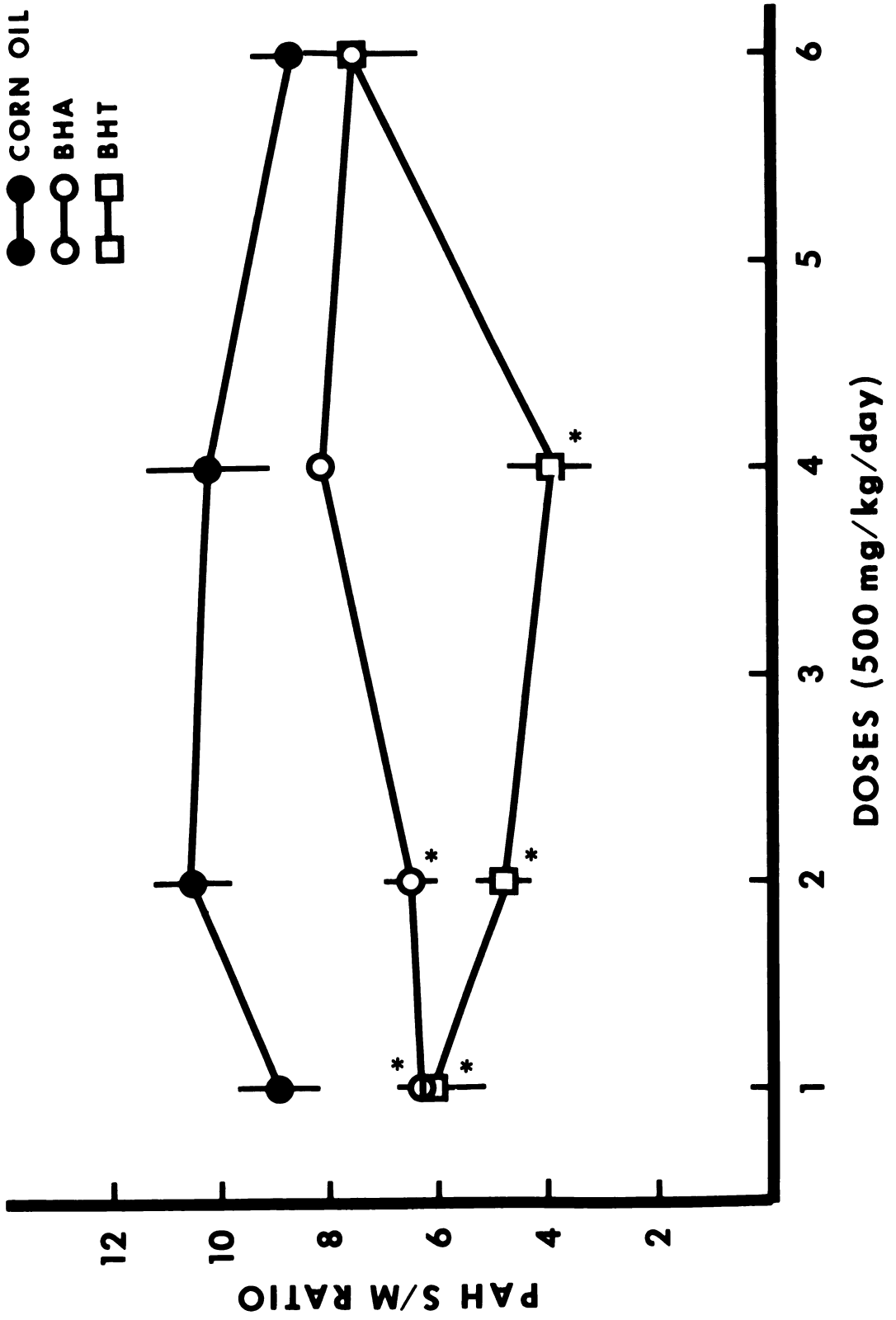

CORN OIL
BHA
BHT
PAH S/M RATIO
12
10
8
6
4
2
1
2
3
4
5
6
DOSES (500 mg/kg/day)
*

Figure 4

Figure 5. Accumulation of NMN by cortical slices from kidneys of rats treated with butylated hydroxyanisole or butylated hydroxytoluene. Each point represents the mean ± S.E.M. of four experiments. No significant differences were found among the groups.

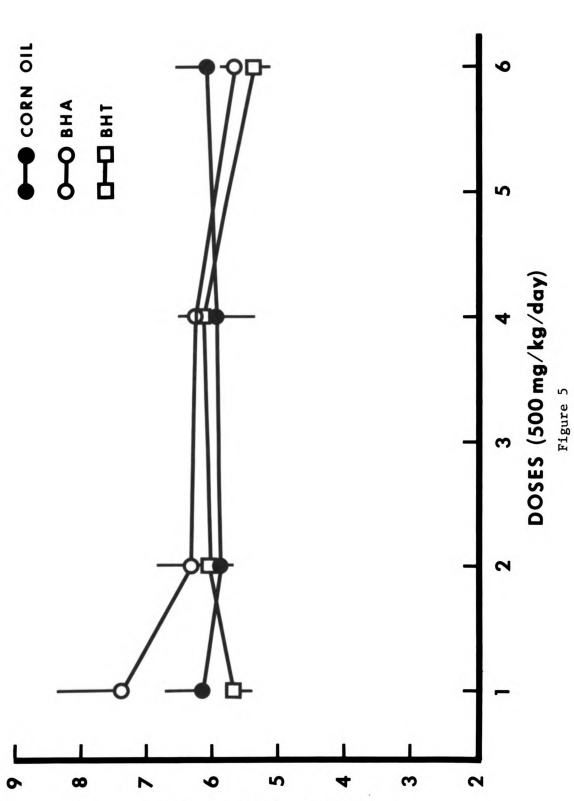

CORN OIL
BHA
BHT
NMN S/M RATIO
9
8
7
6
5
4
3
2
1
2
3
4
5
6
DOSES (500 mg/kg/day)

Figure 5

Fluid balance. No significant changes occurred during the course of the experiment in either the water intake or urine volume of the control or BHA-treated animals (Figure 6). There was a large increase in urine volume in the 24 hours following the second dose of BHT (25.5±3.7 ml) compared to the volume on day 0 (14.3±1.7 ml). No changes in water intake were apparent when compared to control (day 0) values (33.6±0.8 ml), although a significant increase was evident between day 2 (24.3±4.4 ml) and day 4 (40.3±8.2 ml).

Urine composition. The pH of the urine of animals treated with BHA or corn oil did not change significantly during the six days of treatment (Table 2). The urinary pH for the BHT groups however, decreased markedly after two doses, then increased slightly through the sixth day of treatment.

The daily sodium and potassium excretion of control animals did not change significantly for the duration of the experiment (Figure 7). The potassium excretion of the BHA-treated rats decreased from 3.757± 0.417 mEq/day on day 0 to 2.350±0.326 after one dose, and subsequently increased. Administration of BHT resulted in depressed potassium excretion on days 1 and 2. Sodium excretion was reduced significantly by treatment with either agent on days 1, 2, and 4, and by BHA on day 6. The osmolality of the urine from BHT-treated animals was depressed on days 1 through 6; BHA had no effect (Figure 8). The total excretion of osmotically active particles decreased after one and two doses of either antioxidant, but approached control values by day 4.

The toluene in the flasks of many of the rats treated with BHT turned bright pink or amber; that from BHA-treated animals was

Figure 6. Effects of BHA and BHT on water intake and urine volume of treated rats. Each point represents the mean ± S.E.M. of four experiments. Asterisks indicate differences from control (day 0) values ($p<.05$).

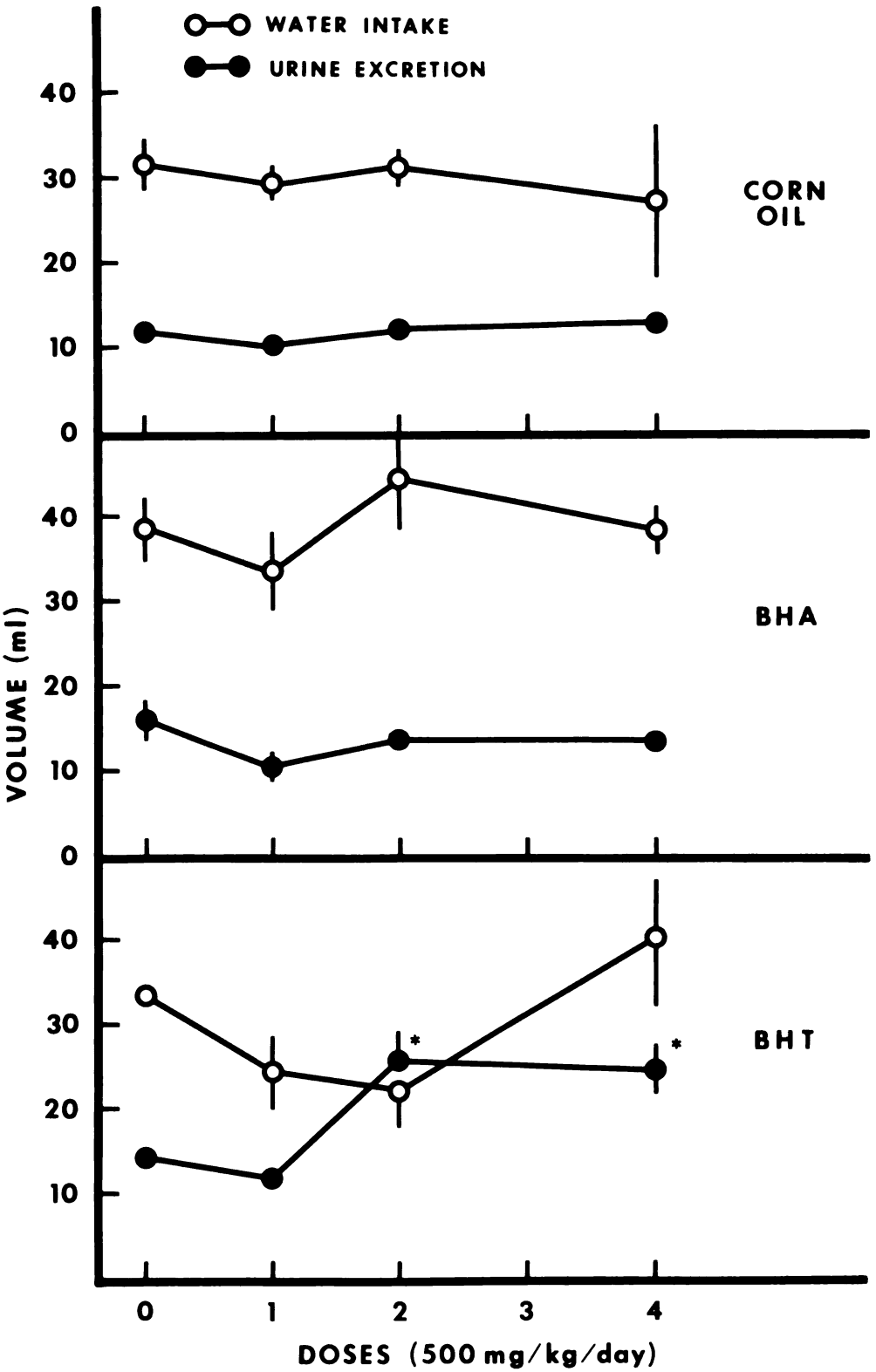

WATER INTAKE
URINE EXCRETION
CORN OIL
BHA
BHT
VOLUME (ml)
40
30
20
10
0
*
*
0
1
2
3
4
DOSES (500 mg/kg/day)

Figure 6

TABLE 2

Effect of BHA or BHT Treatment on the pH of 24 hour Urine Samples

Groups	Urine pH				
	Number of Doses				
	0	1	2	4	6
BHA	7.91±0.29[a]	7.41±0.39	7.31±0.38	7.64±0.14	7.64±0.23
BHT	8.20±0.07	7.51±0.14[b]	6.87±0.18[b]	6.98±0.24[b]	7.42±0.28[b]
Corn oil	8.13±0.11	7.86±0.07	7.91±0.11	7.89±0.24	8.02±0.11

[a]Values represent means ± S.E.M.

[b]Significantly different from day 0, $p<0.05$.

Figure 7. Sodium and potassium excretion of animals treated with 500 mg/kg/day of BHA or BHT for 1, 2, 4, or 6 days. Each symbol represents the mean ± S.E.M. of four experiments. Asterisks indicate significant differences from the corn oil group at each day. The shaded symbols denote significant differences from the corresponding value at day 0 ($p<.05$).

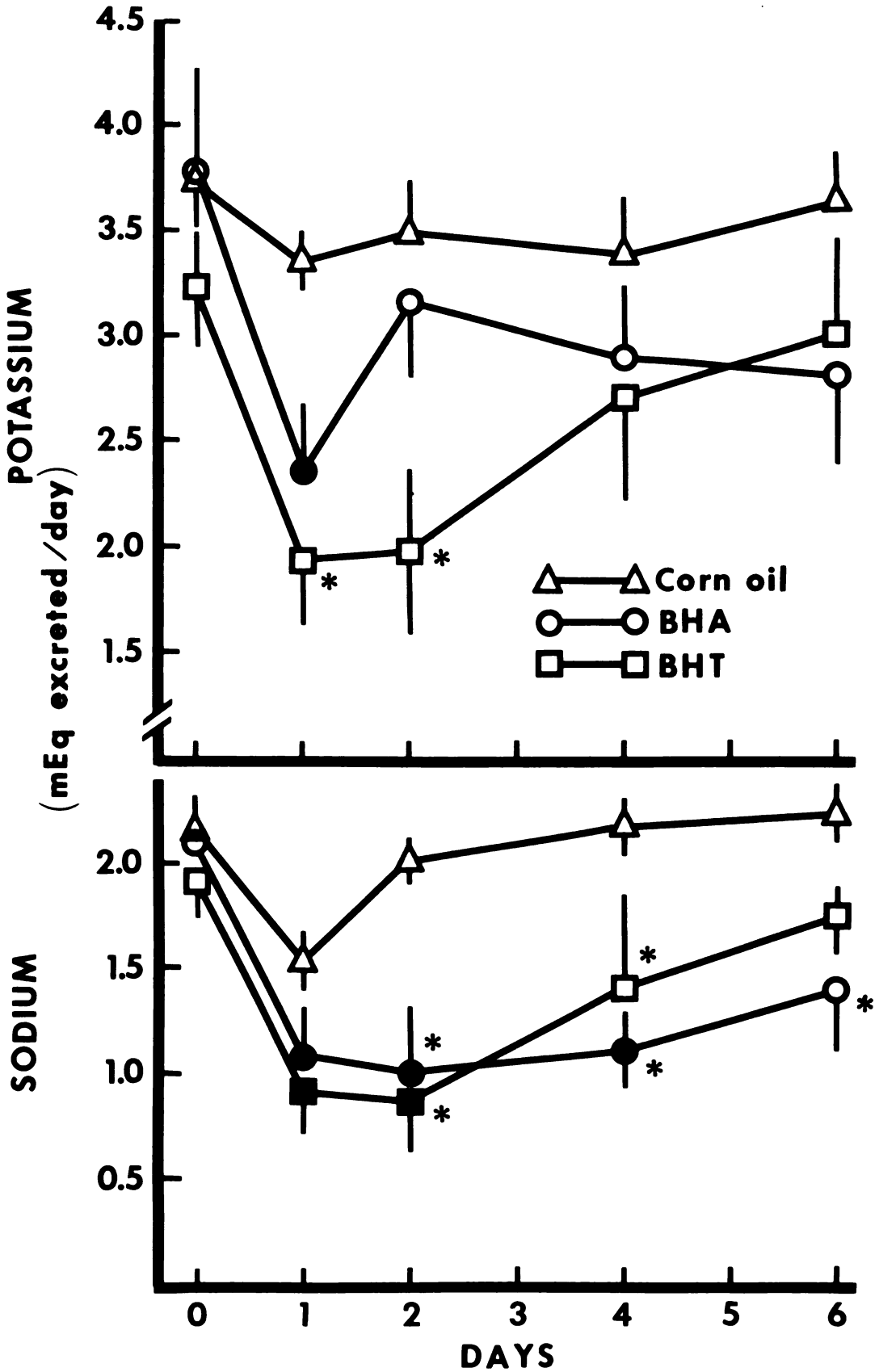

POTASSIUM
(mEq excreted/day)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
Corn oil
BHA
BHT
SODIUM
2.0
1.5
1.0
0.5
0
1
2
3
4
5
6
DAYS

Figure 7

Figure 8. Osmolality and total osmolar excretion in the urine from animals treated with 500 mg/kg/day BHA or BHT for 1, 2, 4, or 6 days. Each symbol represents the mean ± S.E.M. of 4 experiments. Asterisks indicate significant differences from the corn oil group at each day. Shaded symbols denote significant differences from the corresponding value at day 0 ($p<.05$).

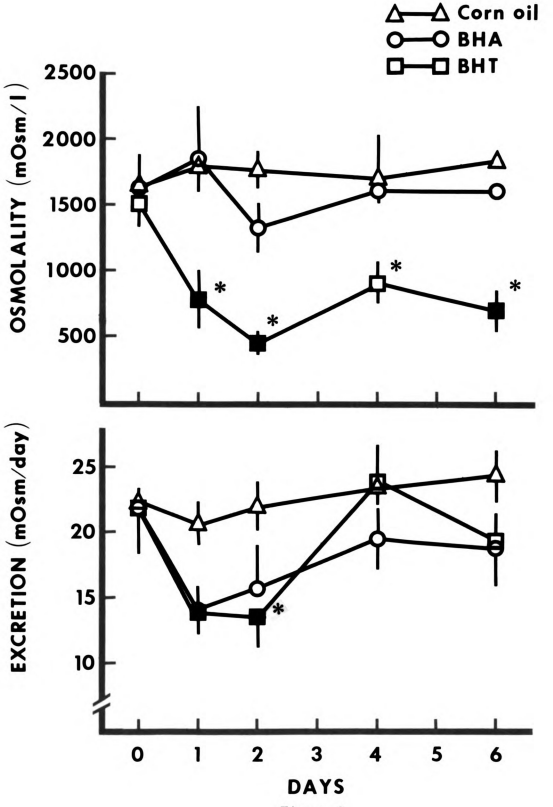

Corn oil
BHA
BHT
OSMOLALITY (mOsm/l)
2500
2000
1500
1000
500
EXCRETION (mOsm/day)
25
20
15
10
0
1
2
3
4
5
6
DAYS

Figure 8

yellow. No color was present in the toluene in the flasks of control animals.

Organ weights. The kidney weights of treated animals (Table 3) were not affected by administration of either antioxidant. BHT treatment produced increases in the weights of the adrenal glands, reaching a maximum of 0.0611±0.078 gm after four doses (Figure 10). BHA did not significantly affect adrenal weights.

Increases in liver weight were noted in animals receiving BHA (Figure 10) which were significantly different after two doses (1.59± 0.60 gm) from corn oil treated rats (13.14±1.04 gm). Despite continued administration of BHA the liver weight of these animals remained at a plateau for the duration of the study. BHT treatment produced a more protracted increase in liver weight, not being significant until day 4, but reaching a greater final magnitude (18.35±1.18 gm) on day 6.

Food intake. The food intake was depressed 38% following one dose of either antioxidant but gradually returned to control levels (Figure 11). With BHT treatment, food intake decreased to a minimum by the second day (6.2±3.0 gm of grain) compared to the corn oil group (23.0±0.84 gm). Subsequently, intakes increased to control levels.

Body weight. BHA treatment did not adversely affect body weight during this study (Figure 12). The weight of animals treated with BHT declined to a minimum after the fourth dose (286±8.9 gm; corn oil, 332±11.0 gm).

Animal appearance. The animals treated with corn oil or BHA seemed alert, with no obvious adverse effects. A number of animals

Figure 9. Daily urinary excretion of sodium, potassium and total osmotically active particles, compared to food intake. Animals received 500 mg/kg/day BHA or BHT for 1, 2, 4, or 6 days, or corn oil alone. All values are expressed as the percent of day 0. Each bar represents the mean ± S.E.M. of four experiments. Asterisks indicate significant differences of the urinary parameter from food intake, at each day ($p<.05$).

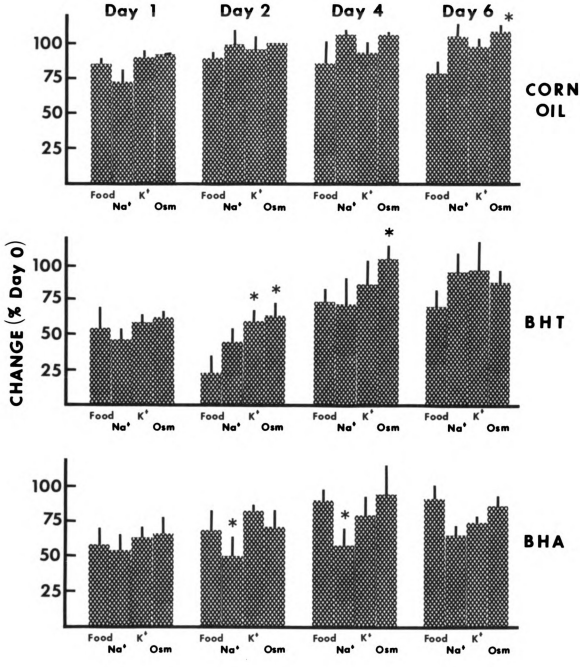

Day 1
Day 2
Day 4
Day 6
CORN OIL
BHT
BHA
CHANGE (% Day 0)
100
75
50
25
Food
Na+
K+
Osm
*

Figure 9

TABLE 3

Effect of Antioxidant Treatment on Kidney Weights

	Kidney Weight (gm)			
	Number of Doses			
	1	2	4	6
BHA	2.46±0.13[a,b]	2.32±0.03	2.36±0.09	2.31±0.03
BHT	2.35±0.05	2.45±0.12	2.22±0.05	2.23±0.10
Corn oil	2.54±0.06	2.39±0.10	2.36±0.07	2.46±0.10

[a]Values represent means ± S.E.M. of four experiments.

[b]No significant differences were found among the treated groups, $p<.05$.

Figure 10. Adrenal weight and liver weight from animals treated with 500 mg/kg/day BHA or BHT for 1, 2, 4, or 6 days. Each symbol represents the mean ± S.E.M. of four experiments. Asterisks indicate differences from the corresponding corn oil group ($p<.05$).

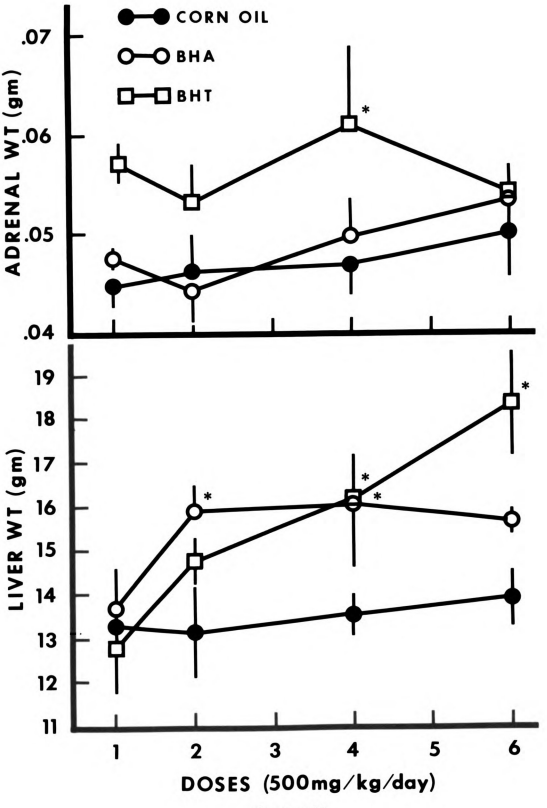

CORN OIL
BHA
BHT
ADRENAL WT (gm)
.07
.06
.05
.04
LIVER WT (gm)
19
18
17
16
15
14
13
12
11
1
2
3
4
5
6
DOSES (500mg/kg/day)
*

Figure 10

Figure 11. Food intakes of animals treated with BHA or BHT for 1, 2, 4, or 6 days. Each point represents the mean ± S.E.M. of four experiments. Asterisks indicate differences from the group receiving the same number of doses of corn oil ($p<.05$).

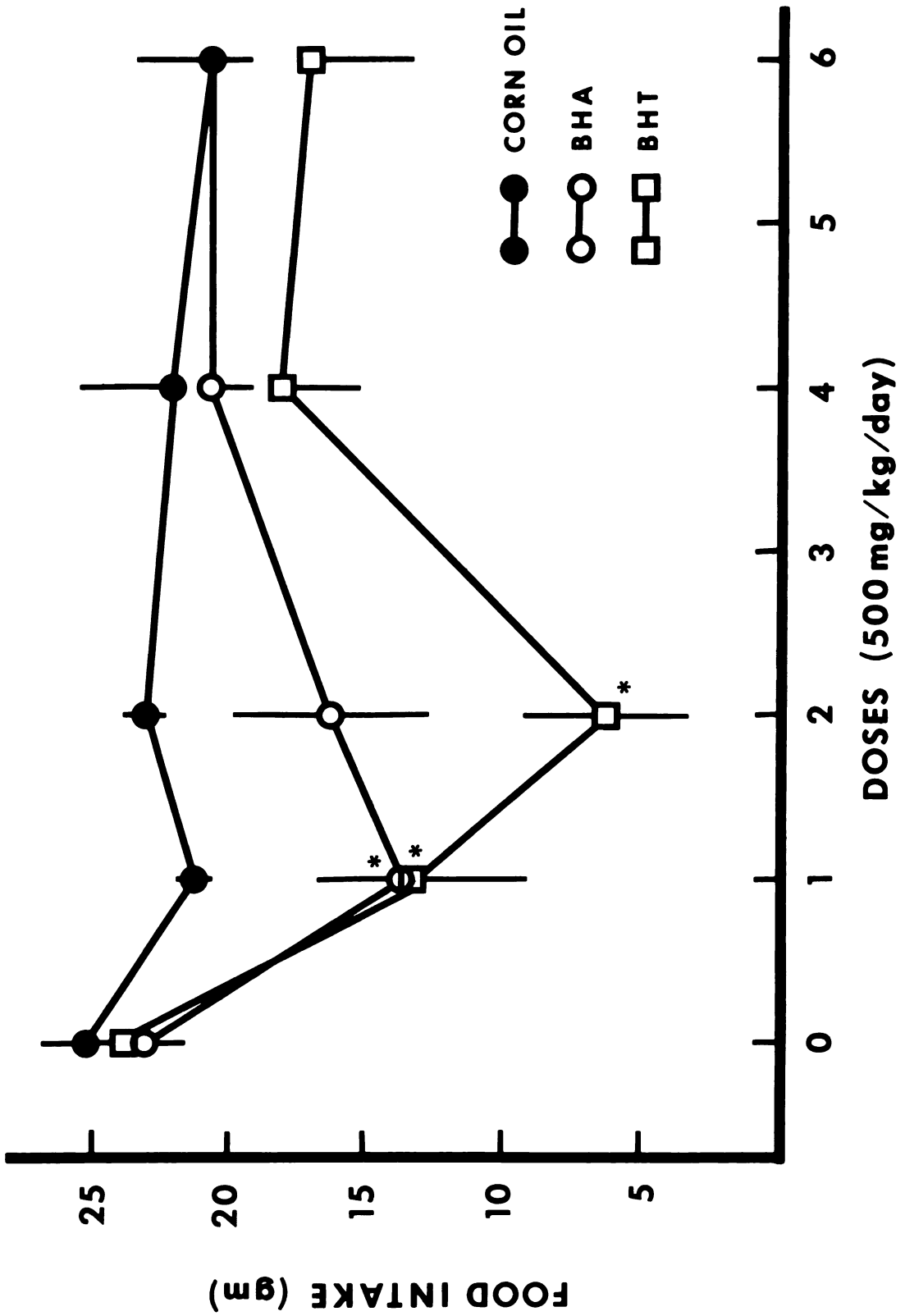

FOOD INTAKE (gm)
25
20
15
10
5
0
1
2
3
4
5
6
DOSES (500 mg/kg/day)
CORN OIL
BHA
BHT
*
*
*
*

Figure 11

Figure 12. Final body weight of animals treated with 1, 2, 4, or 6 doses of BHA or BHT. Each point represents the mean ± S.E.M. of four experiments. Asterisk indicates a difference from the group receiving the same number of doses of corn oil ($p<.05$).

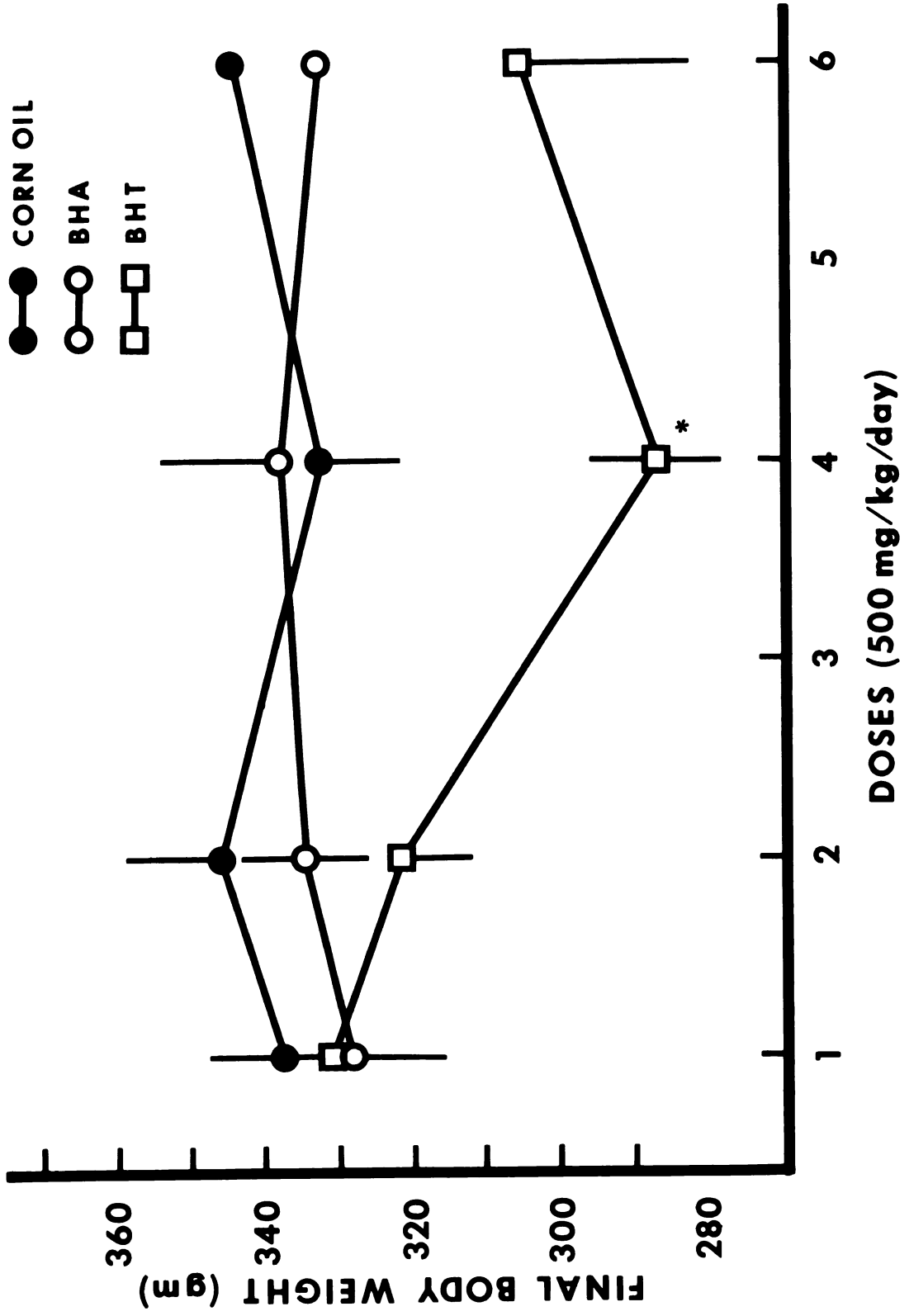

CORN OIL
BHA
BHT
FINAL BODY WEIGHT (gm)
360
340
320
300
280
1
2
3
4
5
6
DOSES (500 mg/kg/day)
*

Figure 12

receiving BHT, however, developed diarrhea with mucous. Many also developed apparent respiratory problems which did not occur in the corn oil and BHA treated rats.

Feeding Study

Organic anion transport. The effects of various levels of food intake on PAH accumulation are shown in Figure 13. The PAH S/M of slices from kidneys of animals pair-fed to control rats remained constant, between 9.30 and 9.43. After one day the S/M from kidneys of animals with restricted food intake (13 gm) was depressed (7.58±0.61). The animals pair-fed to BHA groups then received an additional 3 gm and the S/M increased to control values; the average remained between 9.90 and 9.96 for the next 5 days. The PAH S/M from animals receiving the allotment of the BHT groups was 8.04±0.43 with a grain intake of 6 gm on day 2, and 8.31±0.71 with 9 gm on day 4. On the sixth day the accumulation of PAH had reached control values (9.86±1.42) after 17 gm of food. The S/M of slices from fasting animals were depressed throughout the period of restriction, attaining control ratios only after resumption of food.

Fluid balance. Twenty-four hours after the initiation of fasting, urine volume increased from 16.4±1.4 ml on day 0 to 30.3±8.0 ml (Table 4). However, after two days of fasting a decrease in urine volume occurred, reaching a minimum on day 4 (7.1±2.3 ml). Following resumption of food, the volume increased to control levels (12.6±1.9 ml) on the sixth day. Water intake followed a pattern similar to that of urine volume; decreasing to a minimum on day 4 of fasting (4.6±1.3 ml) and returning to control levels at day 6 (34.0±5.8 ml).

Figure 13. Top: Feeding schedule of animals pair-fed to rats of initial study. Bottom: Effects of restriction of food intake on the accumulation of PAH by renal cortical slices. Each symbol represents the mean ± S.E.M. of four experiments. Asterisks indicate significant differences from the corresponding corn oil group ($p<.05$).

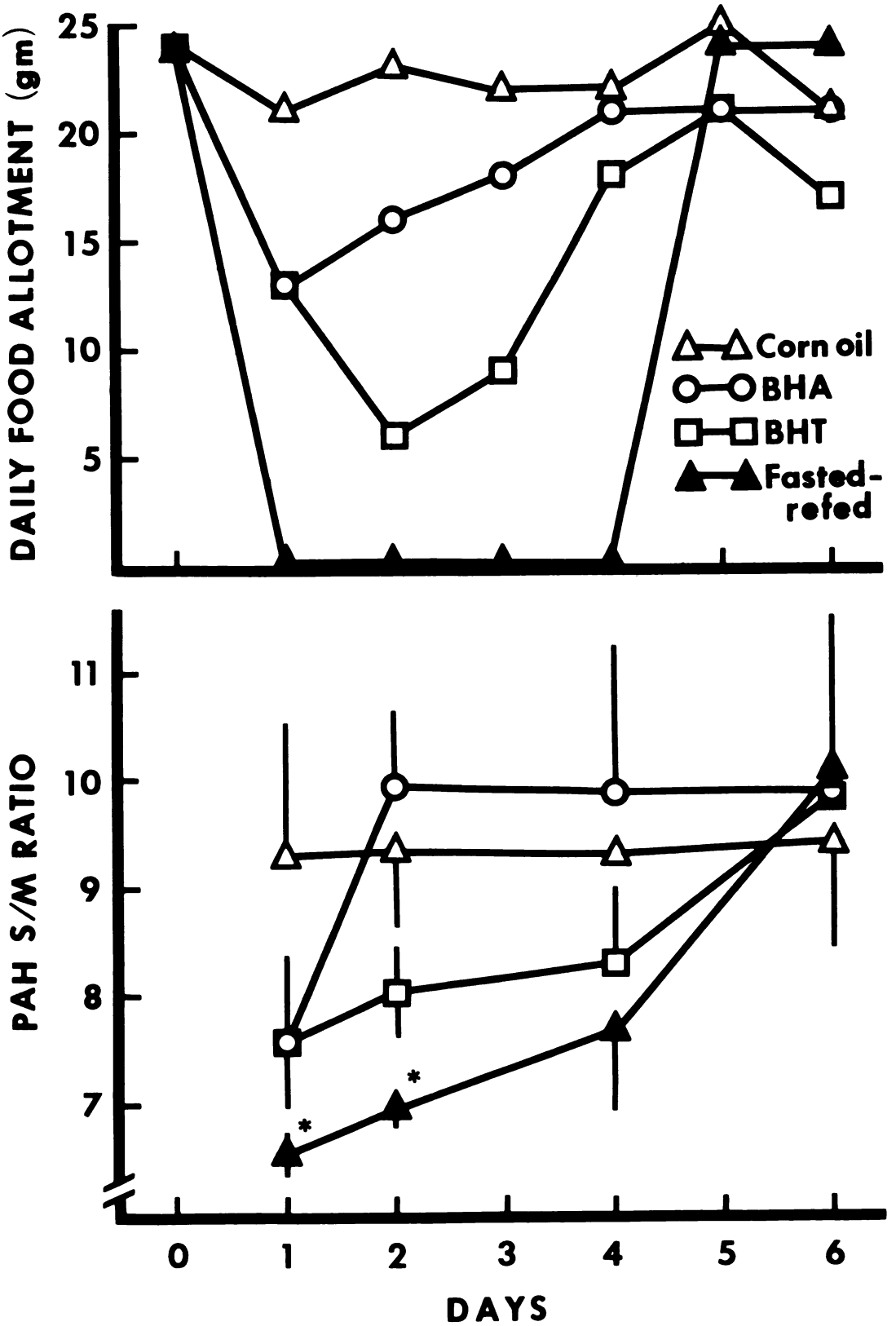

DAILY FOOD ALLOTMENT (gm)
25
20
15
10
5
Corn oil
BHA
BHT
Fasted-refed
PAH S/M RATIO
11
10
9
8
7
*
*
0
1
2
3
4
5
6
DAYS

Figure 13

TABLE 4

Effect of Fasting on Urine Volume and Water Intake

	Fluid Volume (ml)						
	Day of Fasting					Refeeding Period	
	0	1	2	3	4	5	6
Urine volume	16.4±1.4[a]	30.3±8.0[b]	13.9±1.4	6.9±1.9	7.1±2.3	10.7±1.8	12.6±1.9
Water Intake	31.4±1.5	29.0±6.7	17.6±5.8	9.2±2.5[b]	4.6±1.3[b]	32.4±8.5	34.0±5.8

[a]Values represent means ± S.E.M.

[b]Significantly different from day 0, $p<.05$.

In Vitro Study of BHA and BHT

The addition of BHA to the incubation medium of renal cortical slices from untreated rats depressed PAH and NMN accumulation at the two highest concentrations (Figure 14). The slice to medium ratio in both cases was less than 1.0. BHA treatment also resulted in decreased NMN S/M at levels at which the PAH S/M was not affected.

BHT in the medium resulted in a significant depression of PAH transport at the two highest concentrations (Figure 15). NMN accumulation, however, was not affected at any level of BHT.

Enzyme Induction Study

Organic anion transport. The effect of the various treatments on organic acid transport is shown in Figure 16. There were no significant differences between any of the groups.

Enzyme activities. The specific activity of BHT oxidase in saline/corn oil control rats (S/C) was 22.6±2.9 nmoles/hr/mg supernatant protein (Figure 17). Significant increases in activity were obtained with phenobarbital pretreatment, P/C (40.8±2.4 nmoles/hr/mg protein) and BHT treatment, S/B (40.2±3.6 nmole/hr/mg protein). Administration of both agents (P/B) resulted in further augmentation of BHT oxidase activity (55.0±6.4 nmole/hr/mg protein).

Similarly, increases in hexobarbital hydroxylase activity (Figure 17) were noted with phenobarbital (P/C, 6330±674 DPM/hr/mg protein) and BHT (S/B, 7500±612 DPM/hr/mg protein) compared to control animals (4490±510 DPM/hr/mg protein). Treatment with both, P/B, again resulted in a higher level of enzyme activity than did either alone (8163±875).

Figure 14. Effects of addition of BHA to the medium on the accumulation of organic acids and bases by renal cortical slices from untreated rats. Each bar indicates the mean ± S.E.M. of four experiments. Asterisks indicate differences from control media (without BHA) ($p<.05$).

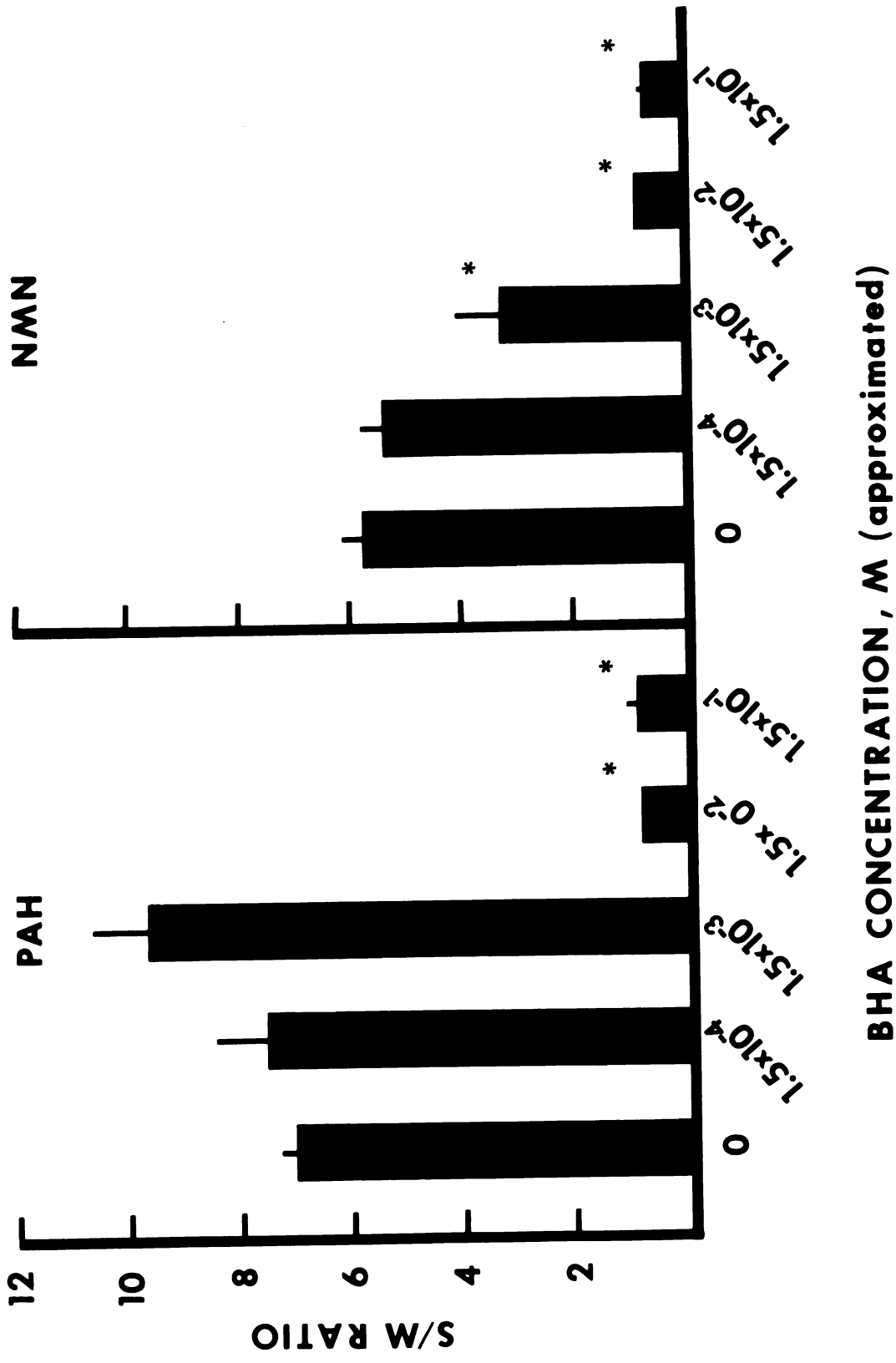

S/M RATIO
12
10
8
6
4
2
PAH
NMN
0
1.5×10⁻⁴
1.5×10⁻³
1.5×10⁻²
1.5×10⁻¹
1.5× 0.2
1.5×10⁻¹
0
1.5×10⁻⁴
1.5×10⁻³
1.5×10⁻²
1.5×10⁻¹
*
BHA CONCENTRATION, M (approximated)

Figure 14

Figure 15. Effects of addition of BHT to the medium on the accumulation of organic acids and bases by renal cortical slices from untreated rats. Each bar indicates the mean ± S.E.M. of four experiments. Asterisks indicate differences from control media (without BHT) ($p<.05$).

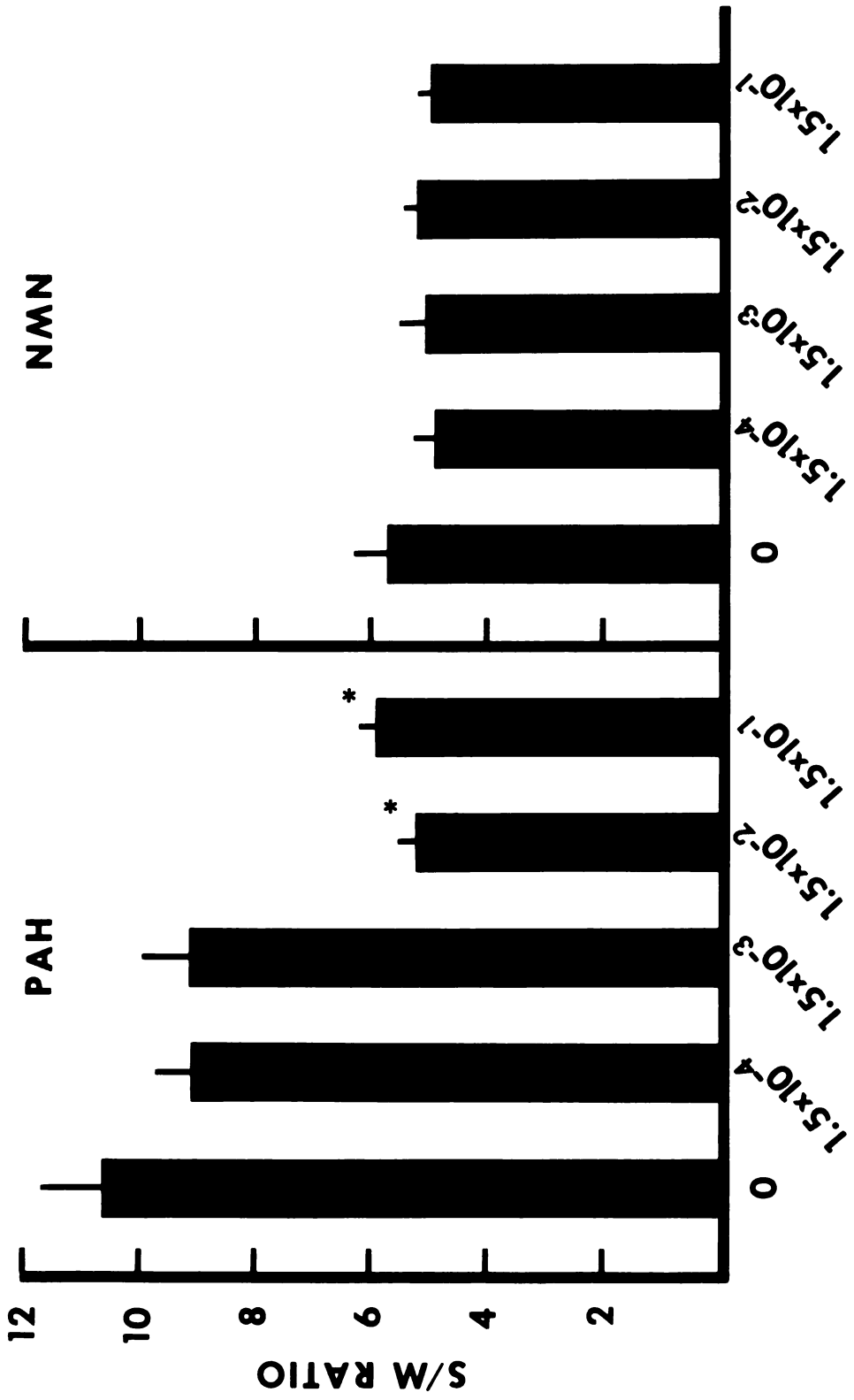

S/M RATIO
12
10
8
6
4
2
PAH
NMN
*
*
0
1.5×10-4
1.5×10-3
1.5×10-2
1.5×10-1
0
1.5×10-4
1.5×10-3
1.5×10-2
1.5×10-1
BHT CONCENTRATION, M (approximated)

Figure 15

Figure 16. Accumulation of PAH by renal cortical slices from animals pretreated with phenobarbital, and subsequently treated with butylated hydroxytoluene. Control groups received saline and/or corn oil. Each bar represents the mean ± S.E.M. of four experiments. No significant differences were found among the groups.

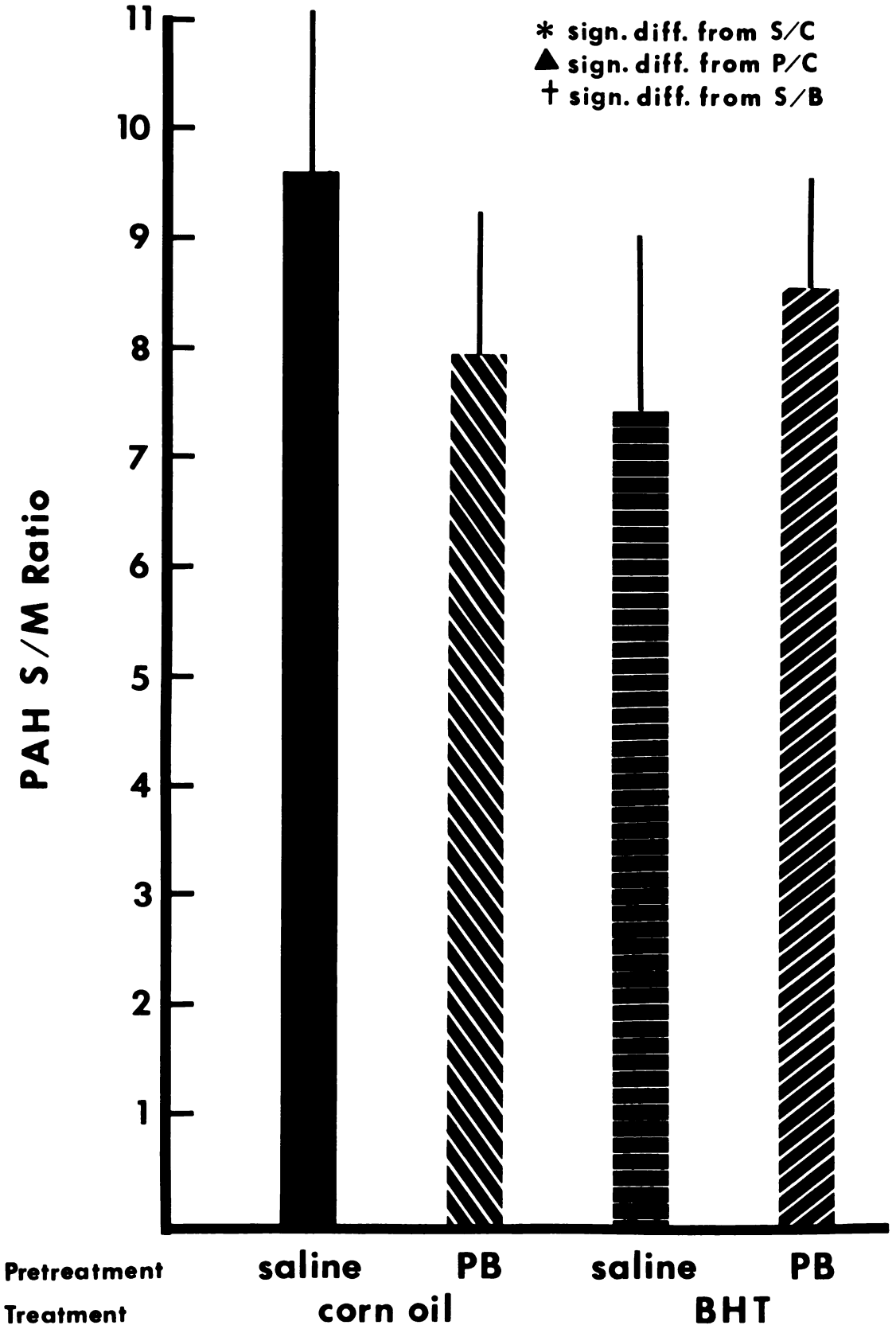

* sign. diff. from S/C
▲ sign. diff. from P/C
† sign. diff. from S/B
PAH S/M Ratio
11
10
9
8
7
6
5
4
3
2
1
Pretreatment
saline
PB
saline
PB
Treatment
corn oil
BHT

Figure 16

Figure 17. Specific activities of hepatic drug-metabolizing enzymes in the 9,000 x g supernatant from rats. Saline and/or corn oil were administered as controls. Each bar represents the mean ± S.E.M. of four experiments.

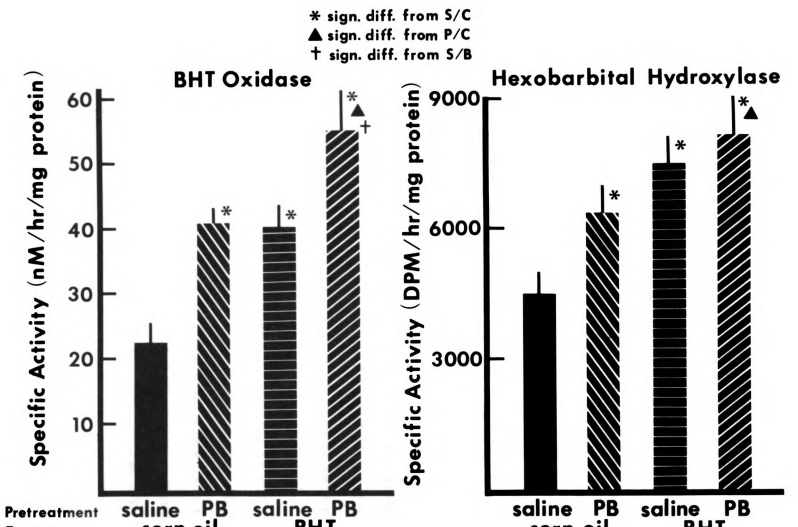

* sign. diff. from S/C
▲ sign. diff. from P/C
† sign. diff. from S/B
BHT Oxidase
Specific Activity (nM/hr/mg protein)
60
50
40
30
20
10
Hexobarbital Hydroxylase
Specific Activity (DPM/hr/mg protein)
9000
6000
3000
Pretreatment
Treatment
saline
corn oil
PB
saline
BHT
PB

Figure 17

Organ weights. As shown in Figure 18, the liver weight of animals treated with corn oil (S/C and P/C) were not significantly different from each other, 8.31±0.30 and 9.77±0.40 gm, respectively. The liver of animals treated with BHT, S/B (19.59±0.46 and P/B (16.61±0.76 gm) were heavier than those from corn oil treated rats. Between the latter two groups the phenobarbital pretreated animals had lighter livers than the S/B rats.

There were no differences in the adrenal weights among the animals treated with corn oil, S/C (51.2±4.2 mg) and P/C (53.7±3.7 mg) and the P/B group (50.2±2.3 mg). As with the livers, the S/B group had heavier organs, the weights of the adrenals averaging 64.0±6.5 mg.

Body weight. During this study there were no effects on body weight of the two pretreatments or two treatments (Table 5). All groups were pair-fed to the S/B groups.

Protein in the liver. A significant depression of the protein concentration of hepatic 9,000 x g supernatants occurred with BHT treatment (S/B, 75.4±1.5 mg protein/gm liver) (Figure 19). There were no differences among the S/C, P/C, and P/B groups.

Increases in the total content of supernatant protein in the livers of P/C and S/B rats were observed, compared to the control, S/C (666±34 mg protein/liver). The content of protein in organs from the BHT-treated groups S/B (1478±62 mg/liver) and P/B (1482±134 mg/liver) were not different from each other, but were both greater than P/C (908±58 mg/liver).

Figure 18. Weight of the liver and adrenals from rats pretreated with phenobarbital and subsequently treated with butylated hydroxytoluene. Saline and/or corn oil were administered as controls. Each bar indicates the mean ± S.E.M. of four experiments.

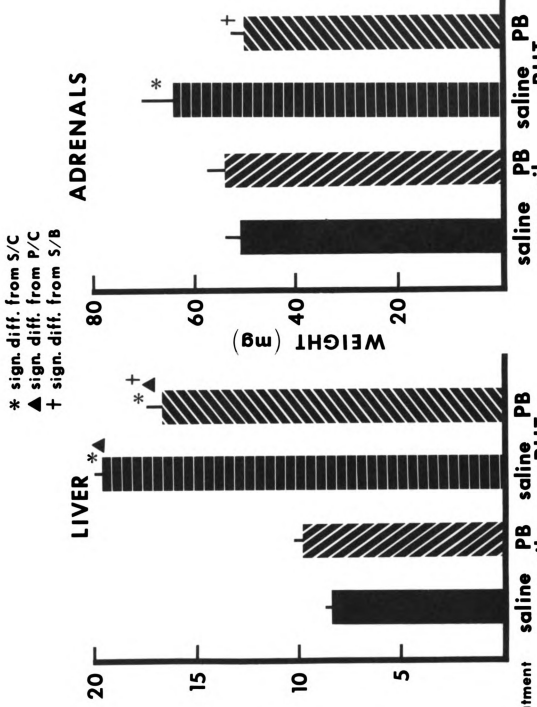

* sign. diff. from S/C
▲ sign. diff. from P/C
+ sign. diff. from S/B
LIVER
ADRENALS
WEIGHT (gm)
WEIGHT (mg)
20
15
10
5
80
60
40
20
Pretreatment
Treatment
saline
corn oil
PB
saline
BHT
saline
PB
saline
BHT

Figure 18

TABLE 5

Body Weights of Animals Treated with Phenobarbital and/or BHT[a]

Pretreatment	Treatment	Initial Body Weight	Post Phenobarbital Body Weight	Final Body Weight
Saline	Corn oil	301±7[b,c]	324±5	295±7
	BHT	306±7	332±3	292±24
Phenobarbital	Corn oil	305±7	339±9	303±9
	BHT	303±4	328±6	293±8

[a]All groups were pair-fed to saline/BHT animals.

[b]Values represent means ± S.E.M.

[c]No differences were found among treatment groups, $p<.05$.

Figure 19. Left: Concentration of supernatant protein in the liver of rats treated with phenobarbital and/or BHT. Corn oil and/or saline were administered as controls. Right: Total content of supernatant protein in the liver of rats exposed to the above protocol. Each bar represents the mean ± S.E.M. of four experiments.

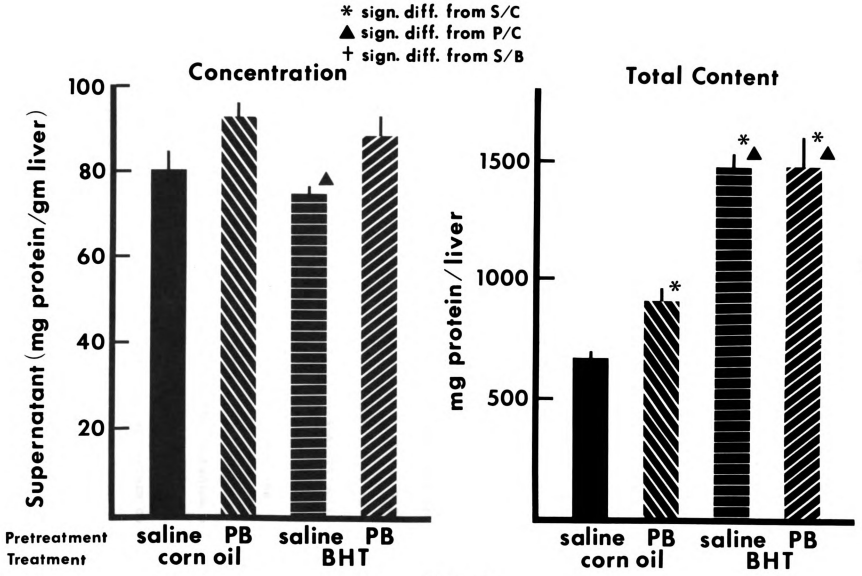

* sign. diff. from S/C
▲ sign. diff. from P/C
+ sign. diff. from S/B
Concentration
Supernatant (mg protein/gm liver)
100
80
60
40
20
Total Content
mg protein/liver
1500
1000
500
Pretreatment
Treatment
saline
corn oil
PB
saline
BHT
PB
saline
corn oil
PB
saline
BHT
PB

Figure 19

DISCUSSION

The study of the effects of foreign compounds on the kidneys--as well as other organs-- is confounded by several philosophical and pragmatic issues. One basic problem is the development of a working definition of a toxicant. A simple criterion such as the production of deleterious effects does not suffice.

With regards to the kidneys, a compound may induce an inappropriate loss of water and solutes. This may be due to a direct effect on the concentrating mechanisms of the nephron; or alternatively, be the result of changes in hemodynamic or humoral factors. In the latter case, although the chemical might be considered harmful, it would not be a nephrotoxin. Infusion of a substance into a laboratory animal might reduce the transport capacity of the kidneys for organic ions; again, this may be due to non-competitive reduction of the transport ability, or conversely to competition for carriers or binding sites. In the latter case, the integrity of the kidneys are maintained and the effects would not be considered toxic. In view of these factors, Foulkes has stressed the importance of establishing the mechanism of action of any compound suspected of being nephrotoxic (75).

Virtually every chemical agent can be harmful under certain conditions, in particular, at a high enough concentration of exposure.

Therefore the dosages of compounds producing equivalent effects, usually death of 50% of a group of animals, have been used as indices of toxicity. This median lethal dose, LD_{50}, is unsatisfactory to describe toxicity inasmuch as the mechanisms of death are seldom noted, and such large doses are often associated with effects on osmolality or food intake which are not observable with lower doses (81). Chronic exposure of laboratory animals to doses approximating the levels ingested by humans is more desirable in order to realistically assess the toxicity of a compound and develop such effects as carcinogenicity. However, when examining the possible adverse effects of a compound for which little information is available regarding pharmacodynamics and interactions with specific biological systems, long-term studies may be wasteful, and subtle changes remain undetected. Short-term studies with dosages high enough to produce morbidity, but not mortality, are therefore important in focusing attention on the physiological systems which are affected and in indicating where further work is needed.

The present study was designed to examine the effects of BHA and BHT on renal function in rats at a dose (500 mg/kg) which is known to produce profound alterations in other organ systems, but without death. The parameters of renal integrity chosen for study were organic ion secretion in the proximal tubule, and fluid and electrolyte balances. With respect to the warning offered by Foulkes (75), attention was given to other factors which might influence the results. The secretion of organic ions was estimated in vitro in order to eliminate extrarenal factors, and food and water intakes, as well as hepatic drug metabolism, were monitored.

The ability of cortical slices from the kidneys of rats treated with BHA or BHT to transport the organic acid PAH was reduced (Figure 4). Based on the model of such transport proposed by Foulkes and Miller (71), several possible explanations may be offered. The processes of organic ion secretion are dependent upon metabolic energy and if the production or utilization of this energy were reduced, it would be expected that transport capacity should be compromised. In this study, organic base accumulation was not affected by either compound (Figure 5); therefore, a non-specific depression of renal metabolism is unlikely. The acidic metabolites of these compounds could be transported specifically by the organic anion system, as are the glucuronide and sulfate derivative of other xenobiotics (82,83). Any untoward effects of these conjugates, however, would be expected to increase in severity as hepatic metabolism is induced and production of conjugates increased. As illustrated in Figure 4, the PAH S/M returned to control values by day 6 despite continued administration.

Fasting has been reported to depress PAH accumulation (84) and it is possible that the observed effects may be due to some factor associated with the decreased food intake (Figure 11). However, the reduction of the PAH S/M noted after four days of food withdrawal (Figure 13) was not of sufficient magnitude to account for the results observed with BHT (Figure 4). In addition, after four doses of BHT the food intake of treated animals was not different from controls (Figure 11), yet at this point the PAH S/M was significantly depressed (Figure 4).

The accumulation of organic anions by renal cortical slices is an index of the transport capacity of the peritubular membrane (71).

Membrane-bound transport mechanisms are sensitive to various factors which may alter the lipid and protein constituents of the phospholipid bilayer (85). BHA and BHT are lipid soluble molecules, and as such are capable of associating with and altering the characteristics of liposomes (14,15). If a physiochemical interaction with the peritubular membrane did occur effects on organic base transport would have been expected as well.

BHA and BHT have been found to bind to proteins and polypeptides such as albumin and bradykinin (13,18,66,67). It is possible that they might exert their effects by interacting with a specific protein involved in the process of accumulation of organic anions (85). Interference with intracellular binding sites for PAH or a carrier located in the membrane would depress the gradient of the ion established by the slices (74). The actions of such transport proteins might also be diminished by inhibition of protein synthesis in the cells. Milner (86) has demonstrated significant reduction of DNA, RNA and protein syntheses in cultured monkey kidney cells within 30 minutes of the addition of 0.034-0.136 mM BHT to the medium. This inhibition was reversed when the medium was replaced, and was not observed with the metabolites BHT-alcohol and BHT-acid.

The hypothesis that the unmetabolized forms of BHA and BHT were the agents responsible for the reduction in organic acid accumulation was tested. Either compound was added to the incubation medium of cortical slices from the kidneys of untreated rats. At the two highest concentrations, BHA was extremely toxic to the tissues (Figure 14). The S/M of PAH and NMN at these levels were less than 1.0. This

implies passive diffusion of the ion, most likely the result of cell death; the slices turned grey and were rubbery and resistant to homogenation. At the lower concentrations, no effects on PAH accumulation were noted. There was a depression of the NMN S/M at 1.5×10^{-3}M BHA.

The addition of BHT to the media produced a decrease in the PAH S/M at the two highest concentrations (Figure 15). Organic base transport, however, was not affected at any concentration. These results suggest that the effects of BHT *in vitro* are specific to organic anion transport, as they are *in vivo*. The differences observed between BHA and BHT *in vitro* may be due to greater toxicity of unchanged BHA to renal tissue, or alternatively to greater solubility of BHA in the incubation medium.

Many of the effects of BHT treatment on laboratory animals are more pronounced than with BHA; as for example the increases in liver weight and induction of drug-metabolizing enzymes noted in several studies (29,39,40). In many cases, the effects of either antioxidant diminish or remain constant despite continued administration. Similar results were found in the depression of the PAH S/M in animals treated with BHA or BHT (Figure 4). The S/M declined to a lower value on the fourth day of BHT administration than it did with BHA. The accumulation of the organic anion by renal slices returned to control values by the sixth day of treatment with either antioxidant; the recovery was faster in the BHA-treated groups than BHT groups.

These results, as well as the observation made by others (29,39, 40), appear to be related to the rate at which the compounds are

metabolized. BHA is conjugated in the liver and the water-soluble metabolites are quickly excreted. BHT, in contrast, undergoes a series of oxidations before being conjugated (12). This factor in addition to enterohepatic circulation, results in delayed excretion of BHT. The protracted elimination is associated with the degree of liver enlargement and induction of mixed function oxidases; presumably the persistance of BHT in the body is a continual stimulus to liver growth (13,34). Consequently, the ability of the animal to metabolize BHT would then be augmented, and any toxic manifestations of the parent compound should be diminished.

The increases in absolute liver weights noted in these experiments (Figure 10) are similar to reports by others who expressed their data as the liver weight/body weight or relative liver weight (RLW). However, the appropriateness of relative organ weights to express toxicological data has been questioned (87,88,89): when the ratio of organ weight to body weight changes only because a marked depression of body weight occurs, errors in interpretation may ensue. In particular, it should be noted that the precipitous fall in body weight of BHT-treated animals in this study (Figure 12) was in part due to the negative water balance (Figure 6).

Detectable increases in the liver weight of rats occurred following treatment with either antioxidant (Figure 10); the effect with BHT treatment was more protracted and of greater final magnitude than with BHA. The liver weight of animals treated with BHA reached a plateau following two doses, and after this time the PAH S/M of these rats began to approach control values (Figure 4). If the agent

responsible for the depression of organic acid accumulation was the unmetabolized form, this phenomenon suggests that after two doses the hepatic ability to metabolize BHA is adequate for the dose presented. The changes in liver weights following 1-4 doses of BHT were comparable to BHA (Figure 10); however PAH accumulation in the BHT-treated rats as lower (Figure 4). The PAH S/M recovered to control values only after the liver weights of the BHT-treated animals were significantly higher than of those treated with BHA. Again, if the presence of unchanged BHT is responsible for the observed effects on PAH accumulation, then it is likely that the hepatic capacity to metabolize BHT is sufficient only after six doses.

It is tenable that the unmetabolized antioxidants may be the agents responsible for the depression of PAH accumulation. Therefore, the hypothesis that induction of hepatic metabolism would reduce the effects on organic anion transport by increasing the production of less toxic metabolites was tested. Animals were pretreated with 100 mg/kg phenobarbital prior to administration of four doses of BHT. No significant differences in the transport of PAH by kidney slices were observed between the groups pretreated with phenobarbital (P/B) and those pretreated with saline (S/B) as shown in Figure 16. Because the group receiving BHT alone (S/B) did not respond to treatment as it had before (Figure 4), no conclusions could be made relative to the hypothesis.

There were striking changes in the livers which indicated that the animals were responding to BHT. The specific activity of BHT oxidase was increased by phenobarbital pretreatment (P/C), as well as

BHT treatment (S/B) (Figure 17). Dual treatment (P/B) produced an even greater effect. The activity of hexobarbital hydroxylase was measured as a positive control, and was found to be similarly induced. BHT treatment for four days did not affect the concentration of protein in the livers of treated rats, although the concentration in S/B rats was less than for those of P/C animals (Figure 19).

The liver from animals treated with four doses of BHT (S/B) were again heavier than controls (Figure 18). Phenobarbital alone (P/C) did not appear to affect liver weight; this was expected since the phenobarbital stimulus had been withdrawn several days previously, and regression of organ weights occurs in such cases (42). The liver weight of animals treated sequentially with phenobarbital and BHT (P/B) were, however, lighter than those from S/B rats.

A similar phenomenon was observed by Schulte-Hermann (34) who noted that the growth of the liver of animals treated with BHT resembles developmental growth: the increased mass is accomplished by hypertrophy as well as hyperplasia. The administration of phenobarbital or SKF 525A in conjunction with BHT reduced the hyperplasia, but not the hypertrophy, in the liver of treated animals. This indicates that the hypertrophic component at least, is independent of alterations in drug-metabolism.

The stimulus to liver growth and induction of drug-metabolizing enzymes following administration of certain xenobiotics has not yet been determined. Presently, considerable investigation is directed toward finding an association with functional load. However, not all inducers of hepatic drug metabolizing enzymes increase liver weights (13,34). It is possible that the stimulus to growth is a by-product

of the xenobiotic metabolism which would be inhibited by SKF-525A. That liver hyperplasia is minimized by phenobarbital might be explained if this agent could induce alternate pathways of metabolism, thereby reducing the production of the postulated stimulus. The effects of phenobarbital pretreatment on the metabolic pathways for BHT have not been examined.

The results of P/B treatment on the weight of the adrenal glands were similar to those of liver (Figure 18): the adrenals of BHT-treated rats (S/B) were heavier than controls, and this was prevented by phenobarbital pretreatment. These results suggest the possibility that some capacity, such as drug-metabolism, exists in the adrenals and is similarly responsive to induction as liver. BHT might also influence the production of a humoral factor which would affect the growth of both organs.

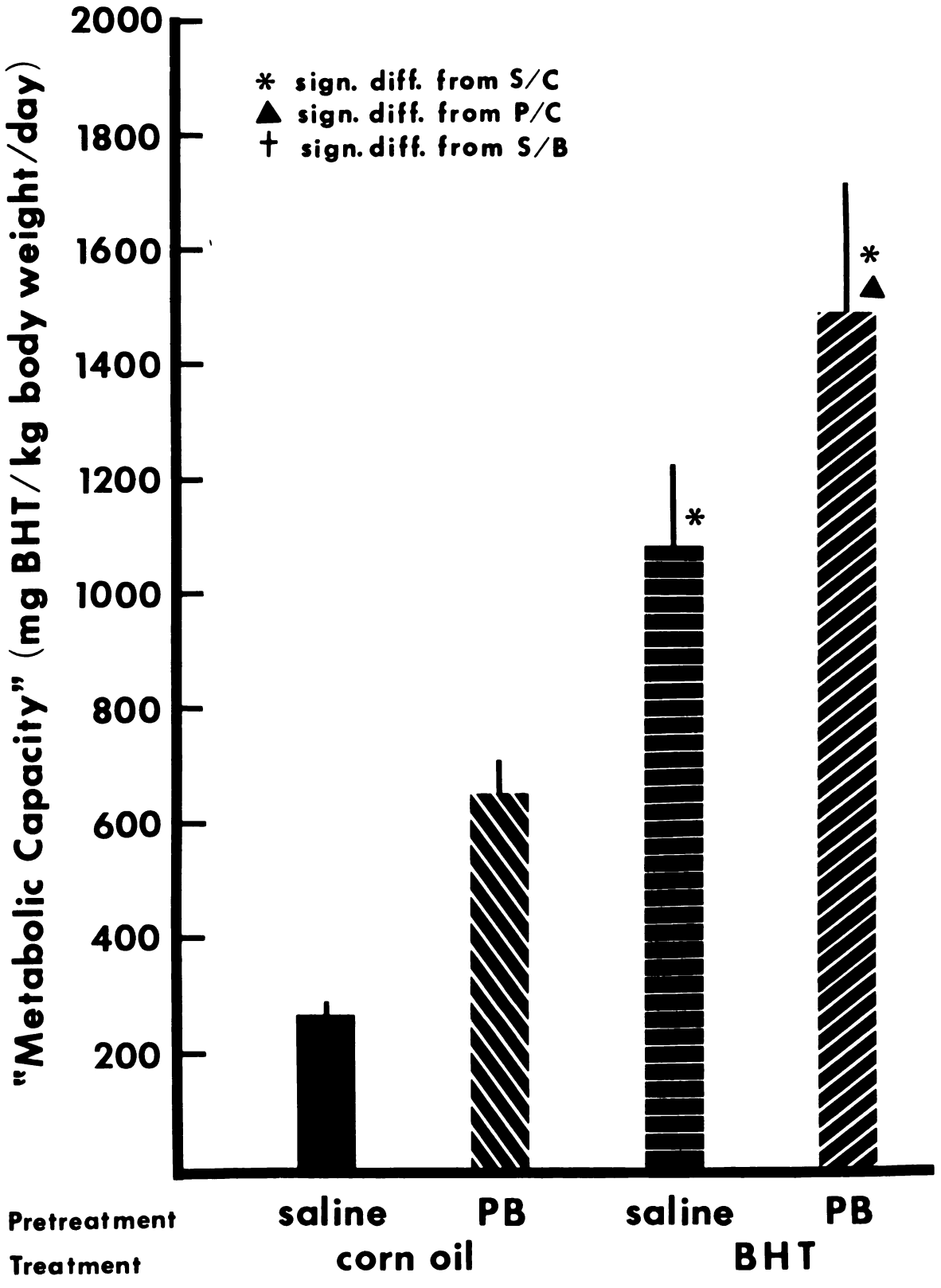
It therefore appears that phenobarbital and BHT treatments stimulated the ability of the rat liver to handle BHT by increasing the amount and activity of an enzyme involved in its metabolism. It is difficult to determine the relative effects in the animal of each treatment regimen by examining the enzyme activities, liver weights, and protein contents individually. In order to consolidate such data, Gilbert and Golberg proposed the concept of metabolic capacity (22). This is a single value which is a function of the protein concentration, specific activity of BHT oxidase, liver weight and body weight, and is expressed as mg BHT/kg body weight/day. This is a biochemical, not a physiological, concept and does not imply that the rat can, or will, metabolize any given amount of BHT in one day. It does not consider

substrate concentrations in vivo, which are affected by the rate of absorption and distribution in body tissues. It does not account for blood flow to the liver, which may be altered by agents such as phenobarbital and subsequently effect substrate availability. The recovery of enzyme activity in the 9,000 x g supernatants or microsomes may be incomplete, and extraheptic sites of metabolism in the intact animals are ignored. Finally, other physiological phenomena that affect reaction rates in vivo, such as circulating hormones, cannot be included in this value. Despite these shortcomings, the metabolic capacity is an index useful for comparing the relative effects of various treatment regimens on the metabolism of BHT.

The metabolic capacities calculated for the animals in the present study are shown in Figure 20, and present cogent evidence that an adaptation occurred in response to BHT and/or phenobarbital treatments which enhanced the ability to metabolize and therefore excrete the antioxidant. Failure to detect the severe depression of PAH accumulation that was observed in previous experiments may simply be a result of the animals in this study responding more rapidly to the antioxidant. Hence, the organic acid transport capacity would have already recovered.

The ability of the kidneys to maintain sodium and potassium balance during administration of either antioxidant was monitored; in contrast to the results of Denz and Llaurado (9), the daily excretions of these electrolytes by animals receiving 500 mg/kg/day BHA or BHT declined during six days of treatment (Figure 7). This discrepancy may be the result of differences in food intakes of the animals in

Figure 20. "Metabolic capacity" for BHT. Animals were pretreated with phenobarbital or saline for three days, then treated with BHT or corn oil for four days. Each bar represents the mean ± S.E.M. of four experiments.


2000
1800
1600
1400
1200
1000
800
600
400
200
"Metabolic Capacity" (mg BHT/kg body weight/day)
* sign. diff. from S/C
▲ sign. diff. from P/C
† sign. diff. from S/B
*
*
▲
Pretreatment
saline
PB
saline
PB
Treatment
corn oil
BHT

these two studies: the former authors indicated that the rabbits receiving either antioxidant failed to eat, whereas in the present study the food intakes of the rats were diminished, but not abolished (Figure 10). Short-term starvation as noted with the rabbits, has been shown to have profound effects on the electrolyte and fluid balance of humans and laboratory animals. Total withdrawal of food induced a natriuresis which was accompanied by a significant increase in urine volume in the initial twenty-four hours (90). Since an increase in water intake did not occur until the second day of fasting , it was not the cause of the diuresis; rather, it appears to be subsequent to plasma volume depletion. This reduction in the plasma compartment was effected on the first day. The osmolality of the plasma from these rabbits was reduced, although no changes were observed in the sodium, potassium, and protein concentrations (90,91). The electrolyte losses are dependent upon caloric deprivation, as a salt-free diet has been shown to produce an adequate conservation of sodium (90,91).

The physiological basis of the natriuresis of fasting has not been determined with certainty. Microperfusion studies in rats fasted for 4-5 days indicate that urine flow and sodium excretion were increased while the clearance of inulin and the excretion of urea were depressed (92). Aldosterone secretion is elevated in the fasting state, even when sodium and potassium intakes are maintained in the drinking water (91). This increase in mineralocorticoid production is therefore not sufficient to prevent the sodium loss accompanying food deprivation, and may explain the increase in aldosterone excretion noted by Denz and Llaurado (9).

A role for the glucoregulatory hormones in electrolyte balance has been proposed. Insulin stimulates retention of both sodium and potassium by the kidneys, whereas glucagon increases the urinary excretion of sodium, potassium, calcium, phosphorous, and magnesium. This is apparently by a direct action on the kidney (93). Since glucagon concentrations in the plasma are elevated during fasting, it is possible that the natriuresis and diuresis observed are mediated through the effects of this hormone.

The rats in the present study were offered distilled water, and housed in wire-bottom metabolism cages. The sole source of sodium and potassium was assumed to be the grain ration, and with adequate renal response the excretion of these electrolytes should be proportional to changes in food consumption. The intake of control animals receiving corn oil alone was depressed approximately 15% for the six days of the experiment (Figure 20); the average dose of corn oil represented about 11% of the normal caloric intake from the grain diet (76). Throughout the study, the daily excretions of sodium and potassium by control animals were appropriate to the food intake, except for a slight excess of osmolar excretion on day 6.

Following one dose of either antioxidant food intakes were depressed by approximately 45%, and the excretion of electrolytes fell proportionately (Figure 9). On the second day of BHT treatment, however, the osmolar excretion, as well as potassium loss, was significantly greater than expected from the intakes. This occurred concurrently with the increase in urine volume (Figure 6). These observations are consonant with the proposed glucagon mechanism for

electrolyte loss, if a 73% reduction of caloric intake would stimulate release of sufficient glucagon. With increased food consumption on days 4 and 6, the excretions of electrolytes again were proportional to intakes. The elevation of urine volume which was still evident on the fourth day was most likely maintained by the polydipsia (Figure 6).

Administration of BHA for six days resulted in potassium excretions concordant with the intakes. Daily sodium output, however was significantly reduced following the second day of treatment. Inasmuch as the water balance of these animals was not affected (Figure 6) and the osmolality of the urine remained constant (Figure 8) a defect in the renal concentrating ability is unlikely.

Renal prostaglandins (PG) increase the rate of urine flow and sodium excretion in laboratory animals (92). BHA has been shown to decrease the production of PGE_2 and PGF_2 by slices of the renal medulla, while BHT had no effect (94). This might offer an explanation of the results in Figure 20; however, in another study infusion of other prostaglandin synthesis inhibitors markedly increased sodium excretion without affecting urine volume or potassium excretion (95). Alternatively, BHA may depress sodium excretion by affecting its absorption in the gut. BHA depressed the absorption of glucose and methionine in rat intestinal preparations; however, the absorption of water was increased (64). This again may be due to the effects of BHA on prostaglandin synthesis, inasmuch as PGs enhance the secretion of water into the intestinal lumen, and block the opposing absorption. Inhibition of PG synthesis in rat jejunum loops with indomethacin decreased secretion of water and increased absorption (96).

Since sodium generally follows the flow of water in the intestines (97), absorption of sodium in the present study would be expected to increase.

After the testing of a food additive to examine its toxic effects, it is important to determine the hazard of the compound for humans. Cassarett (98) has defined hazard as the reciprocal of safety, and safety as "the probability that a substance will not produce damage under specified conditions". BHA and BHT have been shown to have effects on renal function which may be toxic in nature; further work to determine mechanisms of action would clarify this and aid in estimation of the hazard at doses approaching the levels of human intake.

Speculation

Further examination of the interactions of BHA and BHT with other xenobiotics to which humans are exposed accidentally or therapeutically is necessary. BHT-induced stimulation of drug-metabolizing enzymes resembles the broad induction which accompanies phenobarbital treatment (24), and may alter the metabolic pathways for a number of endogenous and exogenous substances. The effects of BHA on prostaglandin synthesis may have important consequences, especially if exposure to other agents which alter prostaglandin metabolism occurs.

SUMMARY

This study was designed to examine the effects of the food antioxidants butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) on selected aspects of renal function. Experiments were designed in order to: 1) determine whether treatment with either antioxidant affects renal secretory processes for organic ions; 2) quantitatively describe the effects of BHA and BHT treatments on renal concentrating ability, particularly with reference to intakes of electrolytes and water; and 3) examine the influences of food intake and induction of drug-metabolizing enzymes on the results.

Adult male, Sprague-Dawley rats were treated with 500 mg/kg/day by gavage of either antioxidant dissolved in corn oil, for 1, 2, 4, or 6 days. During the period of treatment, water intake was monitored, as well as urinary volume, pH, osmolality, and excretion of sodium and potassium. Twenty-four hours following the last dose of BHA or BHT, the rats were killed and the ability of renal cortical slices to accumulate organic ions was determined.

Following one dose of either antioxidant, the food intake of treated animals was depressed. The excretion of sodium and potassium was reduced in proportion to intake. On the second day of treatment with BHT, food intake was reduced further, although electrolyte excretion was not. The observation that excretion of electrolytes was

greater than the intake may be related to impairment of renal function which occurs during severe food restriction. With continued administration of BHT, food intake increased and the electrolyte imbalance was abolished.

Food intake of animals treated with BHA was not significantly different from controls on days 2-5 of treatment. No discrepancies between intakes and urinary excretion of potassium and total osmotically active particles occurred during this period. Sodium excretion, however, was less than expected from the intake. It is not possible to ascertain from these results whether this is due to decreased absorption of sodium in the intestine, or to effects of BHA treatment on the kidneys.

No alterations in fluid intake and output were observed in the rats treated with BHA. Following the second dose of BHT, a large increase in urine volume occurred. Inasmuch as water intake did not increase until the fourth day of treatment, it could not be the cause of the increased urine volume. These phenomena may be related to the natriuresis which occurs with food deprivation as similar results are found in fasted animals.

The ability of cortical slices from the kidneys of treated rats to accumulate a prototype organic anion, p-aminohippurate (PAH), was depressed following one dose of either antioxidant as indicated by the slice to medium ratio (S/M). Despite continued administration of BHA, the PAH S/M was not different from controls following four and six doses. The transport capacity for PAH of renal cortical slices from rats treated with BHT continued to decline, and reached a minimum after

four doses. Following six doses of BHT, however, the PAH S/M was not different from that of controls. The ability of the slices to accumulate the organic base, NMN, was not affected by treatment with either agent.

Fasting for 24 hours has been shown to decrease the ability of slices to accumulate PAH, but not NMN. Because the food intake of the animals in the present study was depressed, an experiment was conducted to determine the contribution of food deprivation to the results. Groups of untreated rats were pair-fed to the animals in the initial study, and killed in the same time sequence. There were no significant effects on PAH accumulation of restriction of food to 50-80% of control intakes. Complete food withdrawal depressed the PAH S/M for the first two days. By the fourth day, however, the difference was not significant. It may be inferred that the effects of antioxidant treatment on organic acid accumulation were not solely due to fasting.

BHA and BHT have been found to induce hepatic drug-metabolism with the subsequent production of water-soluble metabolites that are excreted in the urine. The recovery of renal transport capacity for PAH observed in this study may be due to increased production of such metabolites. Addition of BHA or BHT to the incubation media of slices from the renal cortex of untreated animals indicated that the unmetabolized forms of these antioxidants possess the capacity to depress organic ion accumulation. The effect of BHT *in vitro* was specific for organic acid transport, as found *in vivo*.

The possibility was tested that induction of BHT metabolism contributes to the recovery of organic acid transport. The activity of

BHT oxidase, which catalyzes the rate-limiting step in BHT metabolism, was induced by pretreatment with 100 mg/kg phenobarbital for three days. Four daily doses of 500 mg/kg BHT were then administered and PAH accumulation determined in renal cortical slices. No conclusions could be made relative to the hypothesis, inasmuch as the group pretreated with saline and treated with BHT did not demonstrate a depression of the S/M, as before.

Various changes did occur in the liver which indicated that the animals were adapting to the BHT. The specific activities of BHT oxidase and hexobarbital hydroxylase in the 9,000 x g supernatant were induced by treatment with phenobarbital and/or BHT. The concentration of protein in the 9,000 x g supernatant was not affected by treatment with either agent, although the total content of protein was elevated. The weight of the liver was increased in animals treated with BHT; pretreatment with phenobarbital lessened this effect. A similar phenomenon was noted in the adrenal glands: adrenals were heavier in rats treated with BHT, but not when the animals were pretreated with phenobarbital.

With this series of experiments we have demonstrated that BHA and BHT elicit profound effects in rats which may be renal in origin. Specifically,

-BHT causes production of an increased volume of dilute urine that is not preceded by increased water intake;

-BHA causes a decrease in sodium excretion which may be due to a Na^+ retention, or to decreased absorption in the gut;

-and finally, both compounds depress the renal organic anion transport system.

All of the above effects appear to transient, since various degrees of recovery are noted by the sixth day of the dosing regimen. This phenomenon may be related to increased metabolism, and more experiments are needed on days 2 through 4 of treatment in the period before maximal liver response is attained. Such studies would elucidate the mechanisms responsible for the observed effects, and determine the extent of renal involvement. Investigations with lower doses will aid in making toxicological decisions as to whether these compounds represent a risk in the food supply now, or potentially in the future, as our food habits change and exposure to such foreign chemicals increases.

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