Monograph on **Butylated Hydroxytoluene** 7/24/72

# MONOGRAPH

# ON

# BUTYLATED

# HYDROXYTOLUENE

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BUTYLATED HYDROXYTOLUENE

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# BUTYLATED HYDROXYTOLUENE

## Summary

When BHT is ingested by man, it is readily absorbed and rapidly metabolized. Preliminary oxidation is generally followed by conjugation with glucuronic acid or glycine (13, 30). Elimination of BHT and its metabolites is a biphasic process (14): the first phase is rapid, with 50% of the dose appearing in the urine during the first 24 hours after administration (14); the slower, second phase probably represents the release of BHT or its metabolites from tissue (mainly adipose) storage (14). A biological half-life of 5 days has been determined for the portion of the BHT which is excreted during the second phase (14). Prolonged exposure to BHT does not cause progressive accumulation in the body tissues nor alterations in its handling in man (45).

In rats, BHT consumption has been observed to affect the activity levels of several enzymes. The threshold for increases in liver processing enzyme activity has been found to be between 25 and 75 mg of BHT/kg/day for seven days (23, 24). An intake of 500 mg of BHT/kg/day for seven days causes depression of glucose-6-phosphatase activity and elevation of glucose-6-phosphate dehydrogenase activity (17, 22). No effect was observed on the activity of either enzyme when BHT was given at a dosage of 50 mg/kg/day for 16 weeks (21).

BHT causes an increase in the relative liver weight of test subjects (rats) when given for 16 weeks at a dosage of 50 mg/kg/day in the diet (21). At a dosage of 100 mg/kg/day, there is a slight proliferation of smooth endoplasmic reticulum in liver cells (19). This level of 100 mg/kg/day may be considered the limit of physiologic response, that is, as opposed to toxicologic response.

At dosages of 300 mg/kg/day and greater for 42 weeks, BHT has shown toxic effects: decreased growth rate and exaggerated relative liver and brain weights (19).

Despite administration of 500 mg/kg/day for 7 weeks, no teratogenic effects have been shown for BHT (15). Repeated experiments fail to show any effects on reproductive indices (48), life-span (37), or effects on offspring (7) of a variety of test animals.

At a dosage of 115 mg/kg/day for 8 weeks, BHT results in a decrease of protein synthesis in the liver, a lower liver-N level, and an increase in adrenal weight and adrenal vitamin C content (49).

BHT has shown no carcinogenic activity (5). Indeed, recent studies show that it markedly inhibits the carcinogenic action of several polycyclic hydrocarbons (53, 55).

Summary Table

| Species | Length of Exp. | No Significant Toxic Effects | Min. significant Toxic Effects | Ref. |
|---|---|---|---|---|
| Rat | 16 weeks | 100 mg/kg/day | | 21 |
| Rat | 2 years | 400 mg/kg/day | 500 mg/kg/day | 16 |
| Rat | Multigeneration | 100 mg/kg/day | 300 mg/kg/day | 20 |
| Rat | 8 weeks | 53 mg/kg/day | 115 mg/kg/day | 49 |
| Chicken | 1 year | 156 mg/kg/day | | 48 |

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BUTYLATED HYDROXYTOLUENE

## Chemical Information

I. Nomenclature

A. Common names
butylated hydroxytoluene
BHT
DBPC

B. Chemical names
2,6-di-tert-butyl-p-cresol
4-methyl-2,6-di-tert-butylphenol
2,6-di-tert-butyl-4-methylphenol
3,5-di-tert-butyl-4 hydroxytoluene

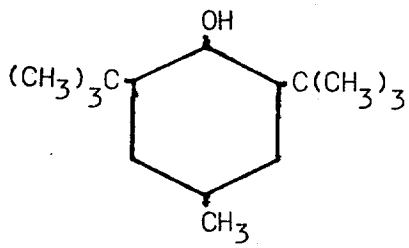
C. Trade names
Ionol
Dalpac
Improvol
Vianol

D. CAS Registry Number - 000128370

II. Empirical formula

$C_{15}H_{24}O_{11}$

III. Structural formula




IV. Molecular weight

220.36

## V. Specifications

A. Chemical
See C.

B. Food Grade
See C.

C. Food Codex
Not less than 99.0% C15H24011
Limits of impurities

| | | |
|---|---|---|
| Arsenic | Not more than | 3 ppm (0.0003%) |
| Heavy Metals | Not more than | 10 ppm (0.001%) |
| Ash | Not more than | 0.002% |

## VI. Description

A. General
white, crystalline, odorless solid

B. Physical properties
melting point 70 deg.
boiling point 265 deg.
insoluble in water
soluble in food oils fats methanol,
ethanol, isopropanol, ethyl methyl ketone,
acetone, cellosolve, petroleum ether,
benzene and most hydrocarbon solvents

C. Stability
0.01% BHT in animal fat reached 20 MEQ Peroxide in 20 hours (control: 4 hrs)
0.02% BHT in cotton seed oil reached 70 MEQ Peroxide in 12 hours (control: 8 hrs)
0.02% BHT in corn oil reached 70 MEQ Peroxide in 15 hours (control: 12 hrs) (50).

## VII. Analytical Methods

A general method for the analysis of BHT and other antioxidants involves the solution of a sample in a suitable hydrocarbon solvent. This solution is then steam distilled and the BHT and BHA are separated from other antioxidants present and analyzed quantitatively. The sample solutions can also be separated by solvent extraction (50).

Quantitative methods depend on a colorimetric determination using a spectrophotometer. In the Emmerie-Engels Method the basis for the color formation is a reduction of ferric chloride reagent to the ferrous state by the antioxidant and the formation of an iron-bipyridyl complex (50). The Szalkowski-Garber Method, the first colorimetric method of analysis specific for BHT, depends upon the orange-red chromogen formed by a reaction involving dianisidine, sodium nitrate and BHT (51).

A systematic method to determine BHT and BHA in the presence of ethoxyquin is based on column chromatography. A sample of the food or feed is dissolved in a suitable hydrocarbon, such as heptane. To determine BHT the solution is passed through a Florisil chromatographic column. All antioxidants and other organic compounds except BHT will remain absorbed on the column. The BHT in the solution is then determined by ultraviolet spectrophotometry (2).

A gas liquid chromatographic technique has been developed which requires about 20 min for the determination of BHA and BHT in vegetable oils (28). Soybean, cottonseed, corn and peanut oils fortified with 20, 60 and 100 ppm each of BHT and BHA showed a recovery range of 97 to 104%.

A thin-layer chromatographic method has been developed which can be used to separate and quantitatively estimate the four principle antioxidants most commonly used in foods. These antioxidants are BHT, BHA, propyl gallate and nordihydroquaiarectic acid. The antioxidants were extracted with 80% ethanol and actonitrile from lard dissolved in n-hexane. After the solvent was evaporated and the residue redissolved in ethanol, the solution was spotted on thin-layer chromatography plates coated with Silica Gel-G. The plates were developed by two-dimensional chromatograph with benzene and acetonitrile and then sprayed with 2,6-dichloroquinone chloride. The antioxidant spots were removed from the plates and prepared for colorimetric determination using appropriate reagents. They were analyzed spectrophotometrically and showed a recovery range of 98 to 101% (47).

## VIII. Occurrence

BHT is a synthetic compound prepared from p-cresol and isobutylene.

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## Biological Data

### I. Acute Toxicity

TABLE I

| SUBSTANCE | ANIMAL | NO. & SEX | ROUTE | DOSAGE | MEASUREMENT | REFERENCE |
|---|---|---|---|---|---|---|
| BHT | Mouse | 5M, 5F | IG | 2000 mg/kg | $LD_{50}$ | 13 |
| BHT | Mouse | 10M | IG | 836-1244 mg/kg | $LD_{50}$ | 13 |
| BHT | Mouse | 5M, 5F | IG | 2500 mg/kg | $LD_{100}$ | 13 |
| BHT | Rat | 4M, 4F | IG | 500 mg/kg | MTD | 13 |
| BHT | Rat | 4M, 4F | IG | 2450 mg/kg | $LD_{50}$ | 13 |
| BHT | Rat | 42M | IG | 1700 mg/kg | $LD_{50}$ | 8 |
| BHT | Rat | 42F | IG | 1970 mg/kg | $LD_{50}$ | 8 |
| BHT | Rat | 4M, 4F | IG | 3500 mg/kg | $LD_{100}$ | 13 |
| BHT | Guinea pig | 3M, 4F | IG | 10,700 mg/kg | $LD_{100}$ | 8 |
| BHT | Rabbit | 6M, 6F | IG | 2100-3200 mg/kg | $LD_{100}$ | 8 |
| BHT | Cat | 9M, 9F | IG | 940-2100 mg/kg | $LD_{100}$ | 8 |

### II. Short-Term Studies

Rats

Three feeding experiments were conducted with a group of 80 white rats. These rats, housed individually, were fed a control diet with a variable casein content (depending on the experiment) or a test diet consisting of the control diet containing 0.2% BHT. Food was provided ad lib, and the experiments were continued for 4-8 weeks (49).

In the first experiment, 16 control rats were fed a stock diet consisting of 19.9% casein, and 16 test rats were fed the same diet containing 0.2% BHT. Growth, food ingestion, and protein efficiency were noted (49).

After 8 weeks, test animals ingested an average of 115 mg BHT/Kg/day, and, at this level, BHT appeared to result in a positive effect on growth, better food ingestion by the rats, and a higher level of protein efficiency in comparison to the control animals (49).

The second experiment involved lowering the casein content of the stock diet to 16.6% for 16 control rats and feeding this stock diet containing 0.2% BHT to 16 test rats for 4 weeks.

After 4 weeks, the final average weight of test rats was 47% greater than that of control animals. A corresponding increase in food ingestion and protein efficiency was evident (49). No data were provided enabling one to calculate the actual mg/kg/day dosage of BHT.

The third experiment was identical to the two preceding experiments except that the casein level was dropped to 9.6%. In this experiment, again, the test animals showed a greater average weight increase (by 41%) than control animals (49).

Biochemical investigations conducted on the test animals after the growth studies revealed that the increase in growth may have been (and probably was) a toxic response. BHT at a 0.2% level in the diet had a negative effect on protein synthesis in the liver. Liver-N content of test rats was significantly lower than that of control rats despite the weight increase of the BHT-fed rats. Liver lipid level, however, as well as adrenal weight and adrenal ascorbic acid content, were increased in the animals fed 0.2% BHT (49).

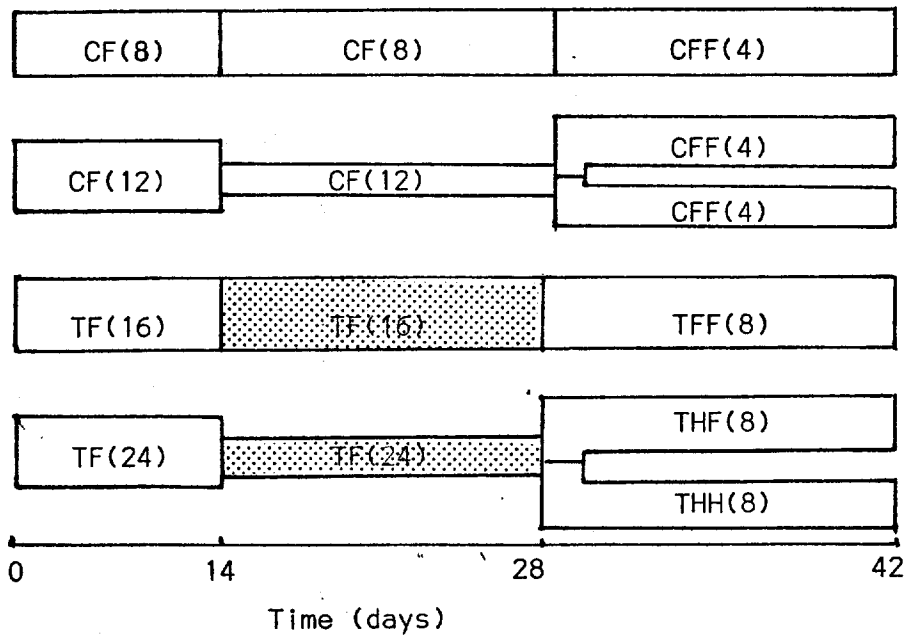
Toxic effects were shown by rats fed BHT at a dosage of 115 mg/kg/day for 8 weeks (49).

Two groups of 48 weanling Carworth SPF rats (24 females, 24 males) were used in a feeding study in which one group was maintained as a control and the other received 0.1% BHT/day in a ground diet. Six rats from each group were sacrificed at 4, 6, 12, and 16 weeks, with daily urine samples being collected in TCA for 4 days before. At autopsy, major organs were weighed and liver samples were taken for biochemical determinations (21) (see biochemical section).

The growth of BHT-fed animals at this level (0.1% BHT/day) closely paralleled that of the control subjects. The only significant changes noted were increase of the organ weight/body weight for liver (females at 4, 6, 12, 16 weeks; males at 4, 12 weeks) and adrenals (females at 12 weeks). Gaunt noted that the presence of BHT in the diet, particularly at high levels (250-500 mg/kg/day), rendered it impalatable to rats and thereby introduced a parameter of stress in growth determination (21).

SPF weanling rats, housed in pairs, were divided into 2 control and 2 test groups as shown:

TABLE II




First letters C, T indicate control and BHT treated respectively. Second letters F, H denote full and restricted diet during treatment phase. Third letters F, H denote same during necessary phase. Shading indicates treatment (500 mg BHT/kg/day) administered by intubation as 10% (w/v) solution in arachis oil.

After a 7 day acclimatization period, data were obtained on urinary ascorbic acid excretion and growth rate from day 7 to day 42. Several biochemical regimens were executed (see biochemical section) (22).

Despite massive dosages of BHT, there was no significant growth retardation. BHT did not affect the relative weight of the adrenals but did increase relative liver weight. This effect was not apparent, however, after the 2 week recovery period (22).

On the basis of studies with hooded rats (Rattus norvegicus), Brown, et al., found that BHT at a dosage of 0.1% BHT/day in a 20% lard-base diet produced a significantly reduced growth rate as well as depilation of the head (6). Subsequent work has failed to confirm this, and consensus among workers is that the effect may have been the result of A-vitaminosis (7).

Chickens

Twenty white leghorn pullets (18 weeks old) were used with 10 held as control animals and 10 fed 0.125% BHT/day in their diet. Three months after pullets came into production, eggs were collected and yolks analyzed for vitamin A. After 4 months of production, the pullets were artificially inseminated and eggs were collected to study the affect of BHT on hatchability. Production records and feed conversion records were kept; liver and blood serum samples were obtained at the end of the experiment (1 year) (48).

At 0.125% BHT/day for 1 year, BHT did not have an adverse affect on fertility, hatchability, viability of offspring nor did it affect the hens in any other apparent way.

Dogs

Four mongrel dogs were fed dosages of 11,200; 8,400; 19,200; and 37,600 mg BHT/kg/day respectively for a 4 week period in a ground meat diet. During the first week, the dogs responded with a mild to moderate diarrhea; during the second and third week, the onset of diarrhea was delayed; and, during the fourth week, only a mild diarrhea developed despite the heavy dosage. No other signs of intoxication were observed; upon autopsy, there was no sign of gastroenteric irritation (16).

A second group of 6 dogs was fed the following dosages of BHT for 260 days over a period of 1 year: 170, 230, 500, 470, 640, and 940 mg/kg. The BHT was mixed in lard and fed with ground meat. At the end of one year, the subjects were sacrificed and histopathological studies of major organs were performed. At the levels of test ingestion, no gross nor microscopic pathology is attributable to BHT (16).

## III. Long-Term Studies

### Rats

For 2 years, 6 groups of 30 Wistar rats (15 females, 15 males) were placed on diets containing 0.2, 0.5, 0.8, and 1.0% BHT/day in 1% lard respectively; one control group was fed 1% lard; and one special group was fed 0.5% BHT/day in 1% lard dissolved and heated for 30 minutes at 150°C. The animals were weighed once a week until a constant weight was attained and monthly thereafter. Blood counts and hemoglobin determinations were executed at intervals (2). At the end of the test period, the rats were sacrificed ($CHCl_3$) and examined for gross micropathologic changes, and organ weights were determined (16).

There was no correlation between mortality and BHT. On the basis of 277 examinations, Deichmann reported no affect on erythrocytes, leucocytes, or hemoglobin levels. There was no effect on weight gain at 0.8%/day; however, at 1%/day both females and males rats manifested a subnormal weight increase. At 0.8%/day there was no effect on organ weight; however, at 1.0%/day both brain and liver of both sexes increased in weight. No consistent pattern of anatomic pathology was observed over the long-term period, even in those animals at the 1.0% BHT/day dosage level (16).

## IV. Special Studies

### Longevity

Two experiments on the effect of BHT on life-span of C57BL mice were carried out. In the first experiment, BHT was administered at 0.7%/day in the diet. Excessive mortality resulted; the dosage was lowered to 0.5%/day and again to 0.2%/day. No conclusion was reached (37).

In the second experiment, BHT was administered at 0.5%/day for 150 weeks. Control mortality was significantly greater than that of the BHT-fed animals. BHT lengthened life-span under sub-optimal conditions and had no significant effect under optimal conditions (37).

### Multigeneration

Three experimental and 2 control groups (16 females, 16 males) of 21 day-old Sprague-Dawley albino rats were fed 20% lard diets containing 0, 300, 1000, and 3000 ppm BHT/day for their entire reproductive life. At 100 days of age, 16 females were mated with 16 males on the same dietary regimen. Fertility, parturition, and lactation were examined in the parents (females) and viability, survival, growth, and behavior in the offspring. At 28 weeks (10 days after weaning the first litter), the females were mated again and the same regimen executed on parents and offspring. At 42 weeks (after weaning of the second litter), these offspring were fed the same diet as their parents until 100 days of age and were mated in the same fashion. The same regimen was again executed on these animals and 2 litters of their offspring (19).

At the 3000 ppm level a 10-20% reduction in growth rate of parents and offspring was observed. Although no effect was noted after 10 weeks, an elevation of serum cholesterol was evident at 28 weeks and a 10-20% weight increase of the liver after 42 weeks. No effects of any kind were noted at the 300 ppm and 1000 ppm levels (19).

All reproductive criteria were normal at all levels of dosage. No teratogenic effects were observed (even at 3000 ppm BHT/day). This multigeneration study substantiated that 1000 ppm BHT/day on a high-fat (20%) diet is the no-effect level (19).

Carcinogenesis

Boutwell and Bosch, using a common pool of mice (C3H, CAF, Sutter) 2-3 months old, performed an experiment determining topical carcinogenicity of various phenol derivatives. One week prior to topical application of test solution, fur was shaved from the test area of the back with electric clippers. As 3% solution of 9,10-dimethyl-1,2-benzanthracene in benzene was applied (1 drop) mid-dorsally as an initiator, and a 20% solution of BHT in benzene was applied (1 drop) twice weekly for 15 weeks. Of 33 test subjects, 28 survived. No papillomas or carcinomas were observed (5).

Inhibition of Carcinogenesis

One group of 18 female Spraque-Dawley rats was given 200 mg BHT in olive oil one hour prior to dosing orally with 12 mg of dimethyl-benzanthracene. A control group of 34 rats was given olive oil and then dosed with DMBA. Eighteen weeks later, 79% of the control group had developed breast tumors; only 28% of the BHT-treated animals had (55).

Further studies using rats and mice as test animals showed that not only did BHT inhibit carcinogenic activity but also inhibited toxic effects attributable to DMBA (55).

In another study, Fischer and Charles River rats were fed 223 ppm N-2-fluorenylacetamide or 239 ppm N-hydroxy-N-2-fluorenylacetamide and 6600 ppm BHT in the diet. BHT reduced the incidence of hepatoma and mammary adenocarcinoma drastically (53).

Teratogenic

Litters of weanling mice (albino and anophthalmic strains) which contained at least 6 females and 1 male were selected and used to comprise 6 test groups, each group containing female and male sibs and 5 unrelated females. Three of the groups were used to test the effect of BHT at 0, 50, 250 mg/kg/day dosages for 18 months in 10% lard; the other 3 groups were used to test the

effect of BHT at 0, 0.1, 0.5% BHT/day for 18 months in 20% lard. The BHT-laden lard was incorporated in the diet. Prior to parturition, females were transferred to separate cages. Pups were observed at day 1, 5, and 12, and the female was returned to the group after 1 week in the nesting cage. This regimen was repeated throughout the reproductive life-span. The experiment was terminated after 18 months (31).

Of 7765 mice born, none were anophthalmic. At 0.1%/day for 18 months, BHT had no statistically significant effect on gestation time, pup weight, litter weight, or litter mortality of the test mice. BHT is not a teratogenic agent (31).

Clegg performed several experiments on rats and mice of various strains. See Table III:

TABLE III

SUMMARY OF ANIMAL GROUPINGS AND DOSAGE

| Exp.* | Strain | No. of Animals | Test Substance | DOSE IU | DOSE mg/kg | Dose regime |
|---|---|---|---|---|---|---|
| | | | | MICE | | |
| 1 | Evans No. 1 | 10 | BHT | | 750 | Daily administration for 32-46 days prior to mating and up to day 18 of pregnancy. |
| 2 | Evans No. 1 | 83 | BHT | | 1000 | Single administration on a specific day of pregnancy (day 1-18). |
| 3 | Evans No. 1 | 10 | BHT | | 750 | Daily administration on days 1-18 of pregnancy. |
| 4 | ICI SPF | 95 | BHA | | 500 | Daily administration for 7 wk before pairing, continuing until day 18 of pregnancy. |
| | | | BHT | | 250<br>300<br>500 | |
| | | | Vitamin A | 30,000 | | |
| | | | | RATS | | |
| 5 | Tuck albino | 32 | BHA | | 750 | Daily administration on days 1-20 of pregnancy.+ |
| | | | BHT | | 750 | |
| | | | Vitamin A | 5,000 | | |
| 6 | Tuck albino | 47 | BHA | | 750 | Daily administration for 70 days before pairing, continuing through pregnancy. |
| | Benger hooded | | BHT | | | |
| | | | Vitamin A | 5,000 | | |
| 7 | Tuck albino | 111 | BHT | | 1000 | Single administration on day 9, 11 or 13 of pregnancy. |
| | Carworth SPF albino | | BHT | | 1000 | |
| 8 | Benger hooded | | Vitamin A | 60,000 | | |
| | Portion SPF albino | 152 | BHA<br>BHT | | 500 | Daily administration for 7 wk before pairing continuing during pregnancy; treatment commenced when rats were 3 or 10 wk old. |
| | | | Vitamin A | 30,000 | | |

*Including arachis oil control and untreated control.
+½ of these rats were allowed to continue to parturition, the offspring being examined at weaning (3 wk old).

BHT was administered in arachis oil by intubation. Except in experiment 6, pregnancies were terminated prior to parturition (day 18 mice, day 20 rats). At termination of pregnancy of the rats, both ovaries and uterus were removed and corporea lutea of each ovary was noted. Number and position of implantation sites (including resorption sites) were recorded and fetuses removed, examined for gross abnormalities, sexed, weighed, fixed, and stained by two procedures for soft tissue examination and for skeletal abnormality examination (7).

This study failed to confirm the findings of Brown, et al. (6). Depilation was not seen and fetal abnormalities were limited to a single case of delayed skeletal development (7).

Dermal

Using rabbits, McOmie, et al., applied BHT topically on gauze to the skin for 24 hours. In another test, BHT dissolved in diethyl ether was flowed on to the shaved back of a rabbit. At the test dosage of 3300 mg/kg for 1 day, the BHT showed no evidence of irritant action. At a dosage of 420 mg/kg in ether solution some erythema and superficial sloughing resulted (43).

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BUTYLATED HYDROXYTOLUENE

## Biochemical Aspects

### I. Breakdown

No information available

### II. Absorption-Distribution

When given orally, BHT is readily and completely absorbed into the intestine. Dissemination of BHT throughout the organism is found when the antioxidant is a regular part of the diet. Being lipid-soluble, the BHT which is deposited is found mainly in the adipose tissue. However, the level of BHT is independent of the duration of dosing and drops back essentially to zero when dosing is terminated, indicating that the BHT deposition is of a dynamic nature.

In extended experiments on rats consuming daily up to 10,000 times the estimated human intake, the BHT level of the rat fat was found to initially rise as high as 200 ppm (days 2-3) and to level off (by day 7) at 100 ppm or less (45).

The rate of absorption and the extent of BHT deposition, at low and intermediate levels of dosage, have been found to be dependent upon sex. The female rat absorbs BHT more readily than the male, resulting in a slightly higher level of BHT deposition in the fat of the female (12, 52).

In chickens given 200 ppm BHT in their feed for 1 to 10 weeks, BHT and its metabolites have been found in the edible portions at levels of 1-3 ppm. The eggs of laying hens fed BHT for 4 weeks contained 2 ppm of BHT and its metabolites (8). In another experiment, dietary levels of 5 and 50 ppm fed to laying hens for 8 weeks caused no accumulation of unchanged BHT in egg fat. When these dietary levels were increased to 100 and 500 ppm BHT, respectively, and the experiment continued for another 21 wks, only a trace of unchanged BHT was found in egg fat at the 100 ppm and 24 ppm BHT at the 500 ppm dietary level (54).

When chickens were fed 0.1% BHT in their diet for eight weeks, an accumulation of 60 ppm BHT was found in the chickens' fat (18).

Botham, et al., fed Alderly Park SPF Wistar rats 5, 50, 500, and 2500 mg/kg BHT in maize oil for periods of 7, 10, or 28 days and thereafter on a BHT-free diet for a recovery period of 2-35 days. At various time intervals test and control animals were sacrificed and microscopic studies and enzymatic assays were run. At 5 mg/kg, no enzyme response could be detected and no proliferation of the smooth endoplasmic reticulum (SER) could be measured. Botham considers the 50 mg BHT/kg level to be a "threshold" value in that this may be at (or near) the level of SER stimulation in the rat. SER proliferation is closely related to the increased activity of aminopyrine demethylase (4).

Nievel performed several experiments with female Wistar rats. In one experiment, rats were given 220 mg BHT/kg in arachis oil by intubation for 1-7 days and sacrificed. In another experiment, dosage procedure was identical except for BHT levels of 15, 50, 150, 450, and 800 mg BHT/kg/day. The capacity of rat liver microsomes to incorporate labelled amino acids was depressed at 15 mg/kg and increased up to 450 mg/kg. At 800 mg/kg the level of incorporation was still increased but less dramatically. Hepatomegaly associated with BHT ingestion results from an increase in and increased nucleoli and is not necessarily indicative of toxicity of BHT (46).

Female specific pathogen free Alderly Park rats (Wistar derived) were given 0.01, 0.1, 1.0 and 5% BHT in their diet for periods of 7-10 days. There was a prompt and rapid increase in SER proliferation in response to the 0.1, 1.0 and 5% BHT levels in the diet. Upon cessation of the dietary BHT, the amount of SER fell off rapidly, except in the case of the 5% level where a much more gradual decline was observed. When rats were given 0.01, 0.1 and 1.0% BHT in their diet for 30 days, at the 1% level there was a large progressive increase of SER proliferation. After withdrawal of BHT, there was a marked reduction of the SER, returning to normal within 15 days. In rats given 1% BHT in their diet for 10 days, allowed a 20 day recovery period, then given a subsequent feeding for a further 10 days the SER response to the second feeding was prompt and of the same magnitude of that observed with the initial feeding, suggesting that no change in the rat's ability to deal with BHT had been induced (9).

Feuer, et al., working with groups of Porton SPF rats found that, when fed BHT in olive oil by intubation at 50, 100, 200, and 500 mg/kg daily for 7 days, rats' relative liver weight was increased significantly at 100, 200, 500 mg BHT/kg levels. However, the activity of glucose-6-phosphatase was depressed significantly only at 500 mg BHT/kg, and glucose-6-phospate dehydrogenase was exalted significantly only at 500 mg BHT/kg (17).

Gilbert and Golberg fed Carworth SPF rats 500 mg/kg/day BHT in arachis oil by intubation. They observed the induction of an enzyme, BHT oxidase. At the dosage in the experiment (500 mg BHT/kg/day) the activities of the following enzymes were markedly increased: aniline hydroxylase; hexobarbitone oxidase; BHT hydroxylase; nitroanisole O-demethylase; and aminopyrine N-demethylase. A figure is given, based on in vitro studies, that the metabolic potential for rats is 42 mg BHT/kg/day (25).

In both male and female rats (Carworth Farm SPF) which were given, by oral intubation, 125 or 500 mg BHT kg/day an increase in relative liver weight and in liver processing enzyme activity was observed. In a further study, rats given 500 mg BHT/kg/day for 1, 2, 3, 4, 7, 14, and 21 days showed a significant increase in hexobarbitone oxidase after 1 day and in relative liver weight, nitroanisole demethylase and aminopyrine demethylase after 2 days. By day 4, relative liver weight and enzyme activity had reached a maximum. In another experiment in which female rats were given doses of 10, 25, 75, 200 and 500 mg BHT/kg/day for one week the threshold dose for liver enlargement and for increased activity of aminopyrine demethylase and hexobarbitone oxydase was determined to be between 25 and 75 mg BHT/kg/day for 7 days (24).

Gaunt, et al., found that rats given 50 mg BHT/kg/day showed increases in liver and adrenal weight and in urinary ascorbic acid excretion (males at 4, 6, 16 wks; females at 4, 12 wks). Histochemical and biochemical examinations showed no significant effect of the BHT upon acid phosphatase, glucose 6-phosphatase, and glucose-6-phosphate dehydrogenase (21). (See biological section for procedural data).

In another study by Gaunt, et al., rats given 500 mg BHT/kg/day for 14 days showed an increase in relative liver weight, but no change in the ascorbic acid content of the adrenals. BHT stimulated the activity of aminopyrine demethylase, nitroanisole demethylase and hexobartitone oxidase. Liver glucose-6-phosphate was severely depressed but returned to control levels after the recovery period. Glucose-6-phosphate dehydrogenase was unaffected. BHT-treated groups had greatly increased serum cholesterol and phospholipid levels (22). (See biological section for experiment procedure).

Adult rats (six male and six female in each group), which had been reared from weaning on diets that were or were not supplemental with 20% of BHT-free lard or of lard containing 0.5% dietary BHT, were injected with acetate-1-(14)C (20 micro-curies/100 g body wt) to study the effect of BHT on lipid metabolism. The rats were sacrificed either 1 hr or 3 hrs after injection, and the expired $CO_2$ and the lipids from various tissues were isolated and chemically and radiochemically estimated. It was observed that BHT, with or without the dietary lard, increased

the basal metabolic rate, the (14)CO2 which expired during the first hour after injection, the concentration of body cholesterol and the rate of synthesis of body and liver cholesterol, and reduced the total fatty acid content of the body. In the rats fed BHT without lard, an increase in the rate of synthesis and turnover of body and liver fatty acids and a reduction in the growth rate were observed. In the rats fed BHT with lard, BHT reduced the rate of synthesis of body and liver fatty acids and reduced the growth rate to a greater extent than in the animals without dietary lard. The authors explained these results by postulating that BHT increases the availability of acetyl coenzyme A and the catabolism of fatty acids (32).

Ames, et al., working with female rats on vitamin E-free diets, found that BHT at 156 mg/kg lowered litter efficiency to 91 (control 100) and that at 775 mg BHT/kg there was a drastic weight loss in pregnant females with resultant fetal deaths (3).

In a study in which White Leghorn pullets were given 0.125% BHT in their feed for a period of one year, the vitamin A and carotenoid pigment content of the eggs, liver and blood serum of the treated birds was observed to be higher than that of the controls (48).

In female White Leghorn chicks fed BHT at 500 mg/kg and or 5000 mg/kg for 3 wks, a reticulocytosis was observed at both dosage levels significantly higher than that of the control group. The worker postulated that the reticulocytosis resulted from the BHT-interference with the necessary oxidation of lipid precursors in the maturation process of erythrocytes (42).

In monolayer cultures of rhesus monkey kidney cells, BHT (30 ppm) caused inhibition of RNA and DNA synthesis, thereby causing inhibition of any increase in cell numbers. This effect varies according to sex, with BHT having a greater effect on cells from females. This difference may be the result of more rapid metabolism of BHT in male cells (44).

Very little testing of BHT in man has been done. Attempts to demonstrate induction of a BHT oxidizing enzyme in humans, even at levels well in excess of those encountered in use, have been unsuccessful. It appears that prolonged exposure to BHT does not result in alterations in its handling in man (45).

## V. Drug Interaction

To determine the effect of BHT on fat metabolism, Karplyuk fed male rats 4 mg BHT/kg body weight for 30-35 days. The feeding of fats containing BHT was found to result in a lowering of the iodine number (which indicates the degree of unsaturation of fat) of the liver fats, indicating that BHT inhibits the oxidation of fatty acids in the animal (36).

## VI. Consumer Exposure Information

In 1964, the FDA reported that the intake of BHT for citizens of the United States was 2 mg/day. A 1967 estimate of the consumption of BHT by citizens of the United Kingdom was 1 mg/day (26). A 1961 estimate of BHT consumption by citizens of the U.S.S.R was 2-3 mg/day (27).

Although the conditional acceptable daily intake of BHT set by the Joint FAO/WHO Expert Committee on Food Additives in 1967 is 2 mg/kg body wt/day (33), the provisions of national legislation make the upper limit of intake, assuming that full advantage is taken of all legal tolerances, 4 mg/day/person (24).

The foods in which BHT is found at the highest levels are vegetable oils, animal fats and oils, and foods containing high levels of fats and oils. The level of BHT added to foods is limited to 200 ppm by weight of BHT based on the fat or oil content of the food. BHT has been cleared for use in some specific applications where addition is made based on the total weight of the food (dry breakfast cereals - 50 ppm; potato flakes - 50 ppm; enriched rice - 33 ppm; chewing gum base - 1000 ppm). If other antioxidants are present in the food, then the total level of antioxidants allowed is the same as that for BHT alone (50).

The following tables (II, III, VI and VII) are preliminary reports on the use of BHT in regular foods. Table II shows the means of usage levels both usual and maximum. Table III shows the daily food consumption calculations for the food categories in which BHT is used. Table VI (Eaters Only) shows the daily intakes of BHT when food in each indicated food category is eaten one or more times during any 14-day period. Table VII (Total Sample) shows the intakes when food in each indicated food category may or may not be eaten during any 14-day period. The intakes in these tables were calculated by multiplying the usage levels (Table II) by the food consumption data (Table III) and by a factor of 10 (to convert % to decimal values and grams to mg., or (1/100) x (1000 mg./gram) = 10). For each substance, the daily intakes were calculated at four different levels by using four different combinations of usage level data (Table II) and food consumption data (Table III): (1) Average Intake = usual level of use x mean food consumption; (2) Normal High Intake A = usual level of use x high food consumption; (3) Normal High Intake B = maximum level of use x mean food consumption; (4) Very High Intake = maximum level of use x high food consumption. The designation "Eaters Only" means that the food consumption calculations per individual food category (and from these the daily intakes) were based only on those persons in the total MRCA sample of 12,743 persons who ate food in the specific food category one or more times during the 14-day MRCA survey period (45).

Table II--Preliminary Calculations on the Use of BHT in Regular Foods per Food Category†

| Food Category No. | | Mean Usage Levels (No. of Firms Reporting) | Usual Level, % | (No. of Firms Reporting) | Max. Level, % |
|---|---|---|---|---|---|
| 01 | Baked Goods | (6) | 0.00048 | (6) | 0.00075 |
| 02 | Breakfast Cereals | (6) | 0.00209 | (6) | 0.00282 |
| 03 | Other Grain Products | (6) | 0.00146 | (6) | 0.00172 |
| 04 | Fats and Oils | (21) | 0.01112 | (21) | 0.01326 |
| 05 | Milk Products | (3) | 0.00210 | (3) | 0.00211 |
| 07 | Frozen Dairy Products | (*) | 0.00029 | (*) | 0.00054 |
| 08 | Processed Fruit, Juices | (*) | 0.00010 | (*) | 0.00015 |
| 10 | Processed Meat Products | (5) | 0.00305 | (5) | 0.00420 |
| 14 | Processed Vegetables | (5) | 0.00081 | (4) | 0.00040 |
| 16 | Soft Candy | (7) | 0.00232 | (7) | 0.00284 |
| 17 | Confections, Frostings | (*) | 0.00100 | (*) | 0.00100 |
| 18 | Jams, Jellies, Preserves | (*) | 0.02000 | (*) | 0.02000 |
| 19 | Sweet Sauces, Syrups | (*) | 0.00700 | (*) | 0.00700 |
| 20 | Gelatin, Puddings | (5) | 0.00036 | (5) | 0.00045 |
| 21 | Soups, Soup Mixes | (*) | 0.00193 | (*) | 0.00220 |
| 22 | Snack Foods | (8) | 0.00274 | (8) | 0.00474 |
| 23 | Beverages--Type I | (13) | 0.00007 | (12) | 0.00015 |
| 24 | Beverages--Type II | (*) | 0.00030 | (*) | 0.00050 |
| 25 | Nuts, Nut Products | (8) | 0.00349 | (8) | 0.00524 |
| 27 | Gravies and Sauces | (*) | 0.00034 | (*) | 0.00044 |
| 30 | Hard Candy | (*) | 0.00013 | (*) | 0.00013 |

†These calculations were made on the basis of usage level data submitted by 46 NAS firms, 21 FEMA firms, and 1 subsurvey firm. Many firms participated in more than one survey; therefore, the number of firms cannot be added to obtain an unduplicated total of firms reporting. No reports of any kind were received on the use of BHT in baby foods.

*The asterisk in parentheses indicates that reports were received from only 1 or 2 firms.

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Table III--Preliminary Daily Food Consumption Calculations per Food Category*

| Food Category | | Daily Food Consumption, grams | | | |
|---|---|---|---|---|---|
| | | Eaters Only | | Total Sample | |
| No. | Name | Mean | High | Mean | High |
| 01 | Baked Goods | 137.3 | 203.9 | 137.2 | 203.8 |
| 02 | Breakfast Cereals | 26.8 | 55.5 | 20.0 | 51.8 |
| 03 | Other Grain Products | 34.2 | 67.3 | 27.8 | 61.4 |
| 04 | Fats and Oils | 17.8 | 31.8 | 17.5 | 31.6 |
| 05 | Milk Products | 56.8 | 155.4 | 39.5 | 120.8 |
| 07 | Frozen Dairy Products | 33.7 | 68.6 | 25.6 | 61.7 |
| 08 | Processed Fruit, Juices | 128.8 | 256.9 | 118.3 | 250.6 |
| 09 | Fruit and Water Ices | 11.5 | 23.9 | 0.7 | 2.5 |
| 10 | Processed Meat Products | 79.2 | 130.4 | 78.4 | 130.1 |
| 14 | Processed Vegetables | 85.5 | 143.4 | 85.0 | 143.2 |
| 15 | Condiments and Relishes | 10.9 | 22.9 | 8.8 | 21.2 |
| 16 | Soft Candy | 11.4 | 24.7 | 5.8 | 17.6 |
| 17 | Confections, Frostings | 3.4 | 6.2 | 0.3 | 0.8 |
| 18 | Jams, Jellies, Preserves | 11.0 | 25.3 | 5.7 | 17.7 |
| 19 | Sweet Sauces, Syrups | 12.3 | 24.4 | 6.8 | 17.9 |
| 20 | Gelatin, Puddings | 30.9 | 62.6 | 20.4 | 52.5 |
| 21 | Soups, Soup Mixes | 50.6 | 106.7 | 31.7 | 84.9 |
| 22 | Snack Foods | 2.1 | 4.6 | 1.3 | 3.7 |
| 23 | Beverages--Type I | 142.3 | 318.9 | 104.0 | 277.7 |
| 24 | Beverages--Type II | 204.1 | 504.0 | 28.0 | 58.3 |
| 25 | Nuts, Nut Products | 10.9 | 23.7 | 5.2 | 15.5 |
| 27 | Gravies and Sauces | 13.3 | 26.4 | 8.3 | 21.3 |
| 28 | Dairy Products Analogs | 5.8 | 17.0 | 0.9 | 1.5 |
| 30 | Hard Candy | 4.9 | 10.2 | 0.6 | 1.7 |
| 31 | Chewing Gum | 0.7 | 1.5 | 0.2 | 0.4 |
| 33 | Sugar Substitutes | 0.7 | 1.7 | 0.1 | 0.1 |
| 34 | Instant Coffee and Tea | 143.1 | 266.2 | 121.1 | 259.4 |

*These calculations are based on frequency-of-eating data obtained from the Market Research Corporation of America, and on portion size data obtained from the U.S. Department of Agriculture Spring 1965 Food Intake Survey. "Eaters Only" refers to that portion of individuals in the MRCA sample who ate foods in these food categories 1 or more times during the 14-day MRCA survey period. "Total Sample" refers to all 12,473 individuals in the MRCA survey, many of whom did not eat food in some of the food categories during the 14-day survey period.

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Table VI--Preliminary Calculations on the Daily Intake (mg.) of FUF per Individual Food Category* (Eaters Only)

| Food Category No. | Food Category Name | Average Intake[1] | Normal High Intake: Usual Use/ High Food[2] | Normal High Intake: Max. Use/ Mean Food[3] | Very High Intake: Max. Use/ High Food[4] |
|---|---|---|---|---|---|
| 01 | Baked Goods | 0.659 | 0.979 | 1.030 | 1.529 |
| 02 | Breakfast Cereals | 0.560 | 1.160 | 0.756 | 1.565 |
| 03 | Other Grain Products | 0.499 | 0.983 | 0.583 | 1.158 |
| 04 | Fats and Oils | 1.979 | 3.536 | 2.360 | 4.270 |
| 05 | Milk Products | 1.193 | 3.263 | 1.193 | 3.279 |
| 07 | Frozen Dairy Products | 0.098 | 0.199 | 0.182 | 0.370 |
| 08 | Processed Fruit, Juices | 0.129 | 0.257 | 0.193 | 0.385 |
| 10 | Processed Meat Products | 2.416 | 3.977 | 3.325 | 5.477 |
| 14 | Processed Vegetables | 0.693 | 1.161 | 0.342 | 0.574 |
| 16 | Soft Candy | 0.264 | 0.573 | 0.324 | 0.701 |
| 17 | Confections, Frostings | 0.034 | 0.062 | 0.034 | 0.062 |
| 18 | Jams, Jellies, Preserves | 2.200 | 5.060 | 2.200 | 5.060 |
| 19 | Sweet Sauces, Syrups | 0.861 | 1.708 | 0.861 | 1.708 |
| 20 | Gelatin, Puddings | 0.111 | 0.225 | 0.139 | 0.282 |
| 21 | Soups, Soup Mixes | 0.977 | 2.059 | 1.113 | 2.347 |
| 22 | Snack Foods | 0.053 | 0.126 | 0.100 | 0.218 |
| 23 | Beverages--Type I | 0.100 | 0.223 | 0.213 | 0.478 |
| 24 | Beverages--Type II | 0.612 | 1.512 | 1.020 | 2.520 |
| 25 | Nuts, Nut Products | 0.380 | 0.827 | 0.571 | 1.242 |
| 27 | Gravies and Sauces | 0.045 | 0.090 | 0.058 | 0.116 |
| 30 | Hard Candy | 0.006 | 0.013 | 0.006 | 0.013 |

*Intakes are for adults and children (males + females), ages 2 to 65+ years. Calculations are based on food consumption data (Table III, Eaters Only) for individuals who eat food in these food categories 1 or more times during any 14-day period. Caution: The intakes for the 21 food categories in this table cannot be added to arrive at a combined total daily intake for these substances; for this information, see Table VII. All calculations are based on the assumption that the substance is added to all foods in each category at the levels indicated (either usual or maximum, as in Table I).

[1]Usual Level, % (Table II) X Mean Food Consumption (Eaters Only, Table III) X Factor

[2]Usual Level, % (Table II) X High Food Consumption (Eaters Only, Table III) X Factor

[3]Maximum Level, % (Table II) X Mean Food Consumption (Eaters Only, Table III) X Factor

[4]Maximum Level, % (Table II) X High Food Consumption (Eaters Only, Table III) X Factor

Note: The Factor is 10, which is necessary to convert % (usage levels) into decimal values, and to convert grams (food consumption) into mg. (intake of substance), or (1/100) X (1000 mg./gram) = 10.

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Table VII--Preliminary Calculations on the Daily Intake (mg.) of DHP per Food Category and the Total Dietary* (TOTAL SAMPLE)

| Food Category No. | Food Category Name | Average Intake[1] | Normal High Intake: Usual Use/ High Food[2] | Normal High Intake: Max. Use/ Mean Food[3] | Very High Intake: Max. Use/ High Food[4] |
|---|---|---|---|---|---|
| 01 | Baked Goods | 0.659 | 0.978 | 1.029 | 1.528 |
| 02 | Breakfast Cereals | 0.418 | 1.083 | 0.564 | 1.461 |
| 03 | Other Grain Products | 0.406 | 0.896 | 0.478 | 1.056 |
| 04 | Fats and Oils | 1.946 | 3.514 | 2.320 | 4.190 |
| 05 | Milk Products | 0.830 | 2.537 | 0.833 | 2.549 |
| 07 | Frozen Dairy Products | 0.074 | 0.179 | 0.138 | 0.333 |
| 08 | Processed Fruit, Juices | 0.118 | 0.251 | 0.177 | 0.376 |
| 10 | Processed Meat Products | 2.391 | 3.968 | 3.293 | 5.464 |
| 14 | Processed Vegetables | 0.689 | 1.160 | 0.340 | 0.573 |
| 16 | Soft Candy | 0.135 | 0.408 | 0.165 | 0.500 |
| 17 | Confections, Frostings | 0.003 | 0.008 | 0.003 | 0.008 |
| 18 | Jams, Jellies, Preserves | 1.140 | 3.540 | 1.140 | 3.540 |
| 19 | Sweet Sauces, Syrups | 0.476 | 1.253 | 0.476 | 1.253 |
| 20 | Gelatin, Puddings | 0.073 | 0.189 | 0.092 | 0.236 |
| 21 | Soups, Soup Mixes | 0.612 | 1.639 | 0.697 | 1.868 |
| 22 | Snack Foods | 0.036 | 0.101 | 0.062 | 0.175 |
| 23 | Beverages--Type I | 0.073 | 0.194 | 0.156 | 0.417 |
| 24 | Beverages--Type II | 0.084 | 0.175 | 0.140 | 0.292 |
| 25 | Nuts, Nut Products | 0.181 | 0.541 | 0.272 | 0.812 |
| 27 | Gravies and Sauces | 0.028 | 0.072 | 0.036 | 0.094 |
| 30 | Hard Candy | 0.001 | 0.002 | 0.001 | 0.002 |
| | Totals (mg.) for all categories ..... | 10.373 | 22.688 | 12.412 | 26.727 |

*Intakes are for adults and children (males + females), ages 2 to 65+ years. Calculations are based on food consumption data (Table III, Total Sample) for individuals who may or may not eat food in these food categories 1 or more times during any 14-day period. The intakes for the 21 food categories may be (and have been) added to arrive at a combined total daily intake for the substance. All calculations are based on the assumption that the substance is added to all foods in each category at the levels indicated (either usual or maximum, as in Table I).

[1]Usual Level, % (Table II) X Mean Food Consumption (Total Sample, Table III) X Factor

[2]Usual Level, % (Table II) X High Food Consumption (Total Sample, Table III) X Factor

[3]Maximum Level, % (Table II) X Mean Food Consumption (Total Sample, Table III) X Factor

[4]Maximum Level, % (Table II) X High Food Consumption (Total Sample, Table III) X Factor

Note: The Factor is 10, which is necessary to convert % (usage levels) into decimal values, and to convert grams (food consumption) into mg. (intake of substance), or (1/100) X (1000 mg./gram) = 10.

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## Bibliography - Biochemical Aspects


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# Effect of DPPD, Methylene Blue, BHT, and Hydroquinone on Reproductive Process in the Rat.* (22656)

STANLEY R. AMES, MARION I. LUDWIG, WILLIAM J. SWANSON, AND PHILIP L. HARRIS.
*Research Laboratories, Distillation Products Industries, Division of Eastman Kodak Co., Rochester.*

Although several compounds have been found to prevent or cure certain vit. E deficiency-symptoms, such as brown discoloration of the uterus and cloudy swelling of the convoluted tubules of the kidney, only 2, diphenyl-p-phenylenediamine (DPPD) and methylene blue, have been reported to substitute for tocopherol in preventing resorption-gestation in vit. E-deficient female rats. In our laboratory also, preliminary experiments indicated that methylene blue and DPPD gave slight positive responses under the conditions of our standard vit. E bioassay procedure(1). Consequently, we were led to investigate this apparent vit. E activity further.

*Methylene blue.* The results of vit. E bioassays in which the responses of 5 levels of methylene blue were compared with five levels of standard, *d*-α-tocopherol, showed median fertility doses (MFD) of 36 mg and 0.29 mg respectively. Thus, methylene blue has less than 1% the activity of α-tocopherol. However, during the course of these experiments, Moore, Sharman, and Ward(2) reported that under the conditions of vit. E bioassay in their laboratory, methylene blue was completely negative. The bioassay procedures used at Cambridge and by us were practically identical except for length of time of depletion. Moore used 5 months and we used about 5 weeks. Consequently, the slight positive response we obtained for methylene blue may have been due to its protective or sparing effect on the small, residual quantities of α-tocopherol still remaining in the tissues of our animals after only 5 weeks deple-

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TABLE I. Effect of DPPD* and *d*-α-Tocopheryl Acetate on Reproductive Performance of Vit. E-Deficient Female Rats.

| | Dietary supplement | | |
|---|---|---|---|
| | Negative control, no suppl. | 0.2% DPPD | 0.002% *d*-α-tocopheryl acetate |
| Fertility index (No. of females pregnant to No. mated) | 3/3 | 9/9 | 9/9 |
| Litter efficiency (% of pregnant animals with at least 1 viable fetus) | 0 | 33 | 100 |
| Mortality (No. of females dying at parturition) | | 2 | 0 |
| Mean length of gestation (days) | | 25 | 22 |
| Mean litter size | | 2.3 | 7.8 |
| Viability index (No. of young alive at 3 days to No. born) | | 0/7 | 63/70 |
| Lactation index (No. of young weaned to No. alive at 3 days) | | — | 61/63 |

* Feed grade DPPD.

tion. Quite recently, Christensen, Dam, and Gortner(3) revised their original conclusion (4) since methylene blue has been found to be ineffective as vit. E in preventing fetal resorption if the depletion diet is rigorously freed of tocopherol, thus eliminating the sparing effect of methylene blue on small, residual amounts of tocopherols in the vit. E-low ration. Therefore, methylene blue is not a vit. E substitute for preventing fetal resorption and, furthermore, should be used with caution, according to Moore(5), since at high levels of 0.252% in the diet it caused growth depression and severe damage (fragmentation) to the spleen of male rats. We also observed that all female rats died when supplemented with 100 mg/day of methylene blue.

*DPPD.* Results of a standard bioassay of DPPD in a Tween-water dispersion for vit. E potency showed that under the conditions of our test (5-12 week depletion period). DPPD possessed 7% the activity of the *d*-α-tocopherol standard on a molar basis. Johnson and Goodyear(6) reported that DPPD (0.385 mg/wk) supported reproduction in female rats maintained on a vit. E-low ration from weaning. Subsequent experiments showed(7) that this vit. E-like effect of DPPD disappeared as soon as the animals' tissue stores of tocopherol were used up.

A further test of the apparent anti-sterility potency of DPPD was conducted in which DPPD was fed mixed into the vit. E-low diet and fed to vit. E-deficient female rats beginning 7 days prior to mating. In Table I are shown the results obtained in comparison with a control group treated identically except that the diet was supplemented with 0.002% *d*-α-tocopheryl acetate instead of DPPD. DPPD in the diet (0.2%) was less effective than *d*-α-tocopheryl acetate (0.002%) in preventing typical vit. E-deficiency gestation resorptions, as evidenced by "litter efficiencies" of 33% and 100%, respectively. But more striking was the effect of DPPD on the duration of gestation and on mortality of young. Gestation was prolonged to 25 days from a normal of 22 days, and all of the newborn rats died before 3 days of age.

Other experiments with a vit. E-free diet or a stock diet confirmed these adverse effects of DPPD. Typical results are shown in Table II. Butylated hydroxytoluene (BHT) and hydroquinone (HQ) were also fed to determine whether these antioxidants would show similar toxicity in pregnant animals. Neither BHT nor HQ at the 0.3% level induced the prolonged gestation and mortality characteristic of DPPD. BHT at the 1.55% level caused a drastic weight loss in the pregnant females which was associated with fetal deaths. This response was not unexpected since at this level of feeding the rats were receiving each day about half the single dose $LD_{50}$ of BHT as reported by Deichmann *et al.* (8).

The DPPD effect is more pronounced in animals fed the supplemented "stock diets" 10 days before insemination than in those fed the supplemented "vit. E-low diets fortified with *d*-α-tocopheryl acetate" starting on the day of insemination. Since these experiments were conducted at different times, comparisons should be made with caution. The most

TABLE II. Effect of DPPD* on Reproduction in Female Rats.

| Group No. | Supplement and level in diet (%) | Fertility index | Litter efficiency | Mortality index | Mean length of gestation | Mean litter size | Viability index | Lactation index |
|---|---|---|---|---|---|---|---|---|
| | Vit. E-low diet† + .001% *d*-α-tocopheryl acetate (supplemented diets fed starting on day of insemination) | | | | | | | |
| 1 | Negative control | 14/17 | 100 | 0/17 | 22 | 7.7 | 73/ 85 | 71/73 |
| 2 | DPPD .0125 | 8/11 | 60 | 1/11 | 23 | 6.2 | 18/ 31 | 18/18 |
| 3 | " .0625 | 11/11 | 88 | 0/11 | 24 | 5.1 | 8/ 51 | 6/ 8 |
| 4 | " .313 | 10/11 | 90 | 2/11 | 25 | 6.0 | 0/ 54 | |
| 5 | " 1.55 | 8/11 | 50 | 1/11 | 26 | 7.3 | 0/ 29 | |
| 6 | BHT .0125 | 9/11 | 100 | 0/11 | 22 | 8.7 | 71/ 78 | 70/71 |
| 7 | " .0625 | 11/11 | 100 | 0/11 | 22 | 7.5 | 73/ 82 | 59/73 |
| 8 | " .313 | 11/11 | 91 | 0/11 | 22 | 8.7 | 72/ 87 | 69/72 |
| 9 | " 1.55 | 9/11 | 0 | 0/11 | 22 | | | |
| | Stock diet‡ (supplemented diets fed 10 days prior to insemination) | | | | | | | |
| 1 | Negative control | 16/17 | 88 | 1/17 | 23 | 10.6 | 71/104 | 69/71 |
| 2 | DPPD .0125 | 10/12 | 83 | 0/12 | 24 | 7.9 | 4/ 79 | 3/ 4 |
| 3 | " .0625 | 15/17 | 70 | 5/17 | 25 | 4.9 | 0/ 49 | |
| 4 | " .313 | 8/10 | 60 | 5/10 | 25 | 5.3 | 0/ 16 | |
| 5 | " 1.55 | 11/13 | 33 | 7/13 | 25 | 4.7 | 0/ 14 | |
| 6 | HQ .003 | 10/10 | 100 | 0/10 | 22 | 8.1 | 79/ 84 | 78/79 |
| 7 | " .3 | 10/10 | 100 | 0/10 | 23 | 7.8 | 65/ 76 | 62/65 |

* Feed grade DPPD. † Diet #301 of Harris and Ludwig(9). ‡ Diet of Ames *et al.*(10) except with linseed meal replacing soybean meal and partially hydrogenated vegetable oil (Primex) replacing margarine oil.

sensitive criterion of DPPD feeding, a high incidence of early mortality of young, occurred in both experiments in groups fed 0.0125% DPPD, the lowest level tested. In another experiment, supplementation of the diet with 400.000 I.U./kg of vit. A or 0.1% of *d*-α-tocopheryl acetate failed to alleviate the toxicity. Those young rats which survived this early period of life (3 days) were able to suckle successfully, as evidenced by a normal lactation index. Considerable maternal mortality occurred at levels of 0.0625% DPPD and higher in the stock diet. The abnormality leading to death seemed to be largely mechanical. Gestation was prolonged, in some instances as much as 36%. Probably the fetuses continued to grow *in utero* until, when parturition did occur, the birth process was exceedingly difficult and prolonged. Many of the young were born dead and most of the mothers that died did so during parturition. Other symptoms occasionally observed were vaginal bleeding and prolapse of the uterus. The physiological defect caused by DPPD is probably failure of "the trigger mechanism" which starts the birth process at the proper time.

*Summary and conclusions.* 1) Vit. E. bioassays (prevention of resorption-gestation) of both methylene blue and DPPD indicated a low order of activity. However, this apparent tocopherol-like potency was due probably to a protective or sparing effect on small residual quantities of vit. E in the diet and in the body tissues. Under conditions where the diet was rigorously freed of tocopherol and the depletion period was extended, methylene blue and DPPD were found by other investigators to be completely devoid of vit. E activity. 2) At higher levels of supplementation, both methylene blue and DPPD were toxic. Methylene blue in doses of 100 mg/day killed pregnant female rats. Feeding pregnant rats diets containing DPPD at a level of 0.0125% in the diet resulted in mortality of their young. Higher levels of DPPD induced prolonged gestation and maternal mortality. Neither BHT (0.313%) nor hydroquinone (0.3%) produced these symptoms of toxicity, but BHT at the 1.55% level in the diet resulted in drastic loss of weight and fetal deaths.

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# The Tumor-promoting Action of Phenol and Related Compounds for Mouse Skin*

R. K. Boutwell and Dorothy K. Bosch

*(McArdle Memorial Laboratory, Medical School, University of Wisconsin, Madison, Wis.)*

In preliminary reports repeated applications of phenol (15) and some substituted phenols (4, 5) were stated to be capable of promoting the appearance of skin tumors in mice following a single initiating dose of dimethylbenzanthracene (1). Tumors also developed in mice not exposed to DMBA but treated with phenol alone for long periods of time. Similar findings have been obtained by Salaman and Glendenning (16). In this report, more extensive data are presented on the role of phenol and its derivatives in promoting the formation of both papillomas and carcinomas. Particular attention is given to the effect of levels of phenol, the strain of mice, and the purity of phenol.

## MATERIALS AND METHODS

Young, adult, albino mice from four sources were used: (*a*) those from Arthur Sutter of Springfield, Mo., (*b*) those from Holtzman Rat Co. of Madison, Wisconsin, (*c*) our own colony of mice derived from Sutter mice, and (*d*) the C3H and $CAF_1$ mice obtained from the Roscoe B. Jackson Laboratory, Bar Harbor, Maine. Upon arrival (or at weaning) the animals were housed in screen-bottomed metal cages in air-conditioned animal rooms (75° ± 3° F.). Water and a diet of Purina Laboratory Chow were given ad libitum. Treatments were begun when the mice were 2-3 months of age. In addition to gross observations, body weight curves and survival records provided evidence that the condition of the mice was satisfactory in all experiments reported.

Croton oil[1] and 9,10-dimethyl-1,2-benzanthracene[2] (DMBA) were used as obtained from the supplier. Solvents for the test substances were reagent grade acetone, refluxed with potassium permanganate and distilled, or redistilled thiophene-free benzene. Reagent grade phenol was used, with the exception of one experiment in which the effect of U.S.P. grade phenol was compared with that of purified phenol prepared from the U.S.P. product. The solutions were made on a weight-volume basis, and they were protected from exposure to light and evaporation of the solvents.

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[1] We are indebted to S. B. Penick and Co. for a generous supply of croton oil.

[2] Distillation Products Industries, Rochester, N.Y.

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Phenol was purified in the following manner: The phenol was dissolved in an excess of NaOH, and the alkaline solution was extracted 5 times with redistilled, reagent-grade diethyl ether. Excess sulfuric acid was added, and the phenol was extracted into diethyl ether and dried over magnesium sulfate. Ether was removed by distillation, and the phenol was twice distilled from zinc dust at 179°-181° F.

About 1 week prior to the first application of the test substance, the fur was shaved from the test area of the back of the mice with electric clippers. Because of the possibility of mechanical irritation and damage to papillomas, the mice were not shaved after the experiment was started. The solutions to be tested were applied as a single drop to the mid-dorsal region of each mouse at the times specified in each experiment. Since the droppers delivered a known volume, the amount of solute deposited on the skin could be calculated. The mice were inspected for tumors weekly. Only typical papillomas larger than about 1 mm. in diameter were counted, care being taken to exclude hyperplasias and other miscellaneous lesions. The gross identifications of both benign and malignant tumors were confirmed periodically by microscopic examination.

## RESULTS

*The production of papillomas and carcinomas in mice treated with phenol alone or with a single application of DMBA followed by phenol.*—The mice used in this experiment had been selectively bred for three generations in this laboratory for susceptibility to a single application of DMBA followed by croton oil. They ranged from 2 to 3 months of age at the start of the experiment. Group 1 consisted of 23 male mice, each of which was given a single application of 75 μg. of DMBA (25 μl. of a 0.3 per cent solution of DMBA in benzene); no secondary treatment was given. Group 2 consisted of 23 female mice, each of which received a single application of 75 μg. of DMBA in benzene followed after 1 week by 1 drop of a 10 per cent solution of phenol in benzene (2.5 mg. phenol in each application) twice a week for the duration of the experiment. The third group of 24 female mice was not pretreated with DMBA. The secondary course of 10 per cent phenol was given in a manner identical to and beginning at the same time as that for Group 2. During the first 6 weeks of phenol treatment many of the mice bore wounds and showed reparative hyperplasia, but thereafter only an exceptional wound was seen. Hair loss

occurred in all mice in the area of phenol treatment by the end of the 1st week, and the hair did not regrow.

The promoting property of phenol is illustrated by the curves in Chart 1, in which the incidence of papillomas expressed as the percentage of the surviving mice bearing one or more papillomas is plotted for the first 24 weeks after the single application of DMBA. During this time only one mouse developed a papilloma in the group (21 of the original 23 mice survived) treated once with DMBA without subsequent treatment. A total of three mice bore papillomas at 42 weeks when this group was killed. The mice of Group 2 (DMBA

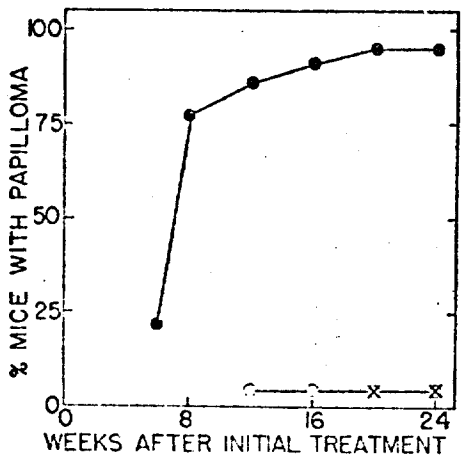



CHART 1.—Tumor promotion by phenol.

*Group 1:* × A single application of 75 μg. of DMBA to the skin of each of 23 mice at zero time.

*Group 2:* ● DMBA as in Group 1, followed after 1 week by twice-weekly applications of 10 per cent phenol (2.5 mg. each) to the skin of 23 mice for the duration of the experiment.

*Group 3:* ○ Twice-weekly applications to 24 mice of phenol only, as in Group 2.

followed by phenol) developed papillomas rapidly beginning 6 weeks after the single application of DMBA, and by the thirteenth week the maximum response of 95 per cent (only 1 negative mouse) was obtained. The average number of papillomas per mouse reached a maximum of 3.0 at the twentieth week and then declined. At this time, 22 of the original 23 mice survived. In contrast, treatment with phenol alone (Group 3) caused tumors to develop slowly; one mouse bore a papilloma at 12 weeks, and at 28 weeks the incidence in 23 survivors was 17 per cent. At 1 year, five of fourteen surviving mice bore papillomas (36 per cent). After 72 weeks phenol treatment was discontinued.

Treatment with phenol also promoted the appearance of malignant tumors. None appeared during 42 weeks in the mice treated once with 75 μg. of DMBA without further treatment. Only one malignant neoplasm was seen during 72 weeks of treatment with phenol alone. It appeared at 58 weeks, and it was identified as a fibrosarcoma. A section is shown in Figure 1. In contrast, many malignancies appeared in the group given the single application of DMBA followed by phenol. The first carcinoma appeared at 19 weeks, at which time the group consisted of 22 mice (effectual total). By 26 weeks 50 per cent of the mice had carcinomas, and at 42 weeks (when this group was terminated) the incidence was 73 per cent of the effectual total.

*The effect of levels of phenol and a comparison with croton oil.*—In this experiment the effect of two levels of phenol (5 and 10 per cent in benzene; 1.25 and 2.5 mg. of phenol per application, respectively) with and without a previous application of 75 μg. of DMBA was tested. For purposes of control and comparison, groups treated with DMBA alone, DMBA followed by croton oil, and croton oil alone were included.

Each group consisted of 30 female albino mice (22 animals in Group 4) obtained from the Holtzman Rat Company as weanling mice. The experiment was begun when the mice were 9 weeks of age, and the treatments were continued for 52 weeks. The mice of Group 1 were given a single application of 75 μg. of DMBA to the skin of the back; no secondary treatment was given. Group 2 received a single application of 75 μg. of DMBA per mouse, and beginning 1 week later each mouse was treated with 25 μl. of a 5 per cent solution of phenol in benzene twice a week for the duration of the experiment. Group 3 received a single application of DMBA (identical to Groups 1 and 2), followed after 1 week by twice-weekly applications of 10 per cent phenol. Group 4 was treated once with DMBA as in the first three groups, and after 1 week each mouse was treated with 25 μl. of a 0.5 per cent solution of croton oil in benzene twice weekly for the duration of the experiment. The mice of Group 5 were given no initial treatment with DMBA; only twice-weekly applications of 5 per cent phenol identical to that used for Group 2 were given. Group 6 was not treated with DMBA; only twice-weekly applications of 10 per cent phenol were given. The mice of Group 7 were treated with 0.5 per cent croton oil in the same manner as was Group 4, but without an initial application of DMBA.

The percentage of mice in each group bearing one or more papillomas is shown in Chart 2A for the first 36 weeks of the experiment. In addition the number of surviving mice at the 24th week

(deaths due to malignancy occurred after 24 weeks) is shown, together with the average weight of each group at this time. The smaller average body weight of the mice treated with 10 per cent phenol and, to a lesser extent, with 0.5 per cent croton oil is indicative of the toxicity of these treatments.

Papillomas appeared rapidly in the mice treated with DMBA followed by 10 per cent phenol (Group 3); in fact, the response was almost identical to that recorded for the mice in which croton oil served as the promoting agent (Group 4). At the 5 per cent level phenol was less effective as a promoting agent (Group 2). Plotting the results in terms of the average number of papillomas per surviving mouse (Chart 2B) revealed greater differences in the promoting activity of croton oil, 10 per cent phenol, and 5 per cent phenol; at 20 weeks the average number of papillomas per mouse in each of these three groups was 6, 3.3, and 0.25, respectively. Papillomas appeared much more slowly in Groups 5, 6, and 7 (secondary treatments alone without initial exposure to DMBA). By 36 weeks the incidence had reached 25 per cent of the mice treated with either 10 per cent phenol or with croton oil, but only one mouse treated with 5 per cent phenol had developed a papilloma. No papillomas resulted from the single application of DMBA alone (Group 1).

The treatments were continued until 52 weeks, and the appearance of carcinomas was recorded (Chart 2C). None developed in the mice treated with DMBA alone nor on those to which either 5 or 10 per cent phenol had been applied repetitively without an initiating treatment with DMBA. Following pretreatment with DMBA a few malignant skin tumors appeared in the mice treated with the 5 per cent solution of phenol. At the 10 per cent level of phenol, the carcinoma incidence reached 47 per cent at 40 weeks and plateaued at that level. The incidence of carcinomas in Group 4 (promoting agent was croton oil) reached 47 per cent at 32 weeks and plateaued at 76 per cent after the 42d week.

The appearance of typical mice treated with 5 and 10 per cent phenol after a single application of DMBA (Groups 2 and 3) is shown at 36 weeks in Figures 2 and 3. There was no evidence of ill effects of repeated applications of 5 per cent phenol except for the development of papillomas in those mice pretreated with DMBA (Fig. 2). In contrast, at the 10 per cent level there was severe damage, including depilation and sebaceous gland destruction, regardless of pretreatment with DMBA. In addition, many papillomas and carcinomas were apparent at 36 weeks in the mice pretreated with DMBA and given 10 per cent phenol twice a week (Fig. 3).

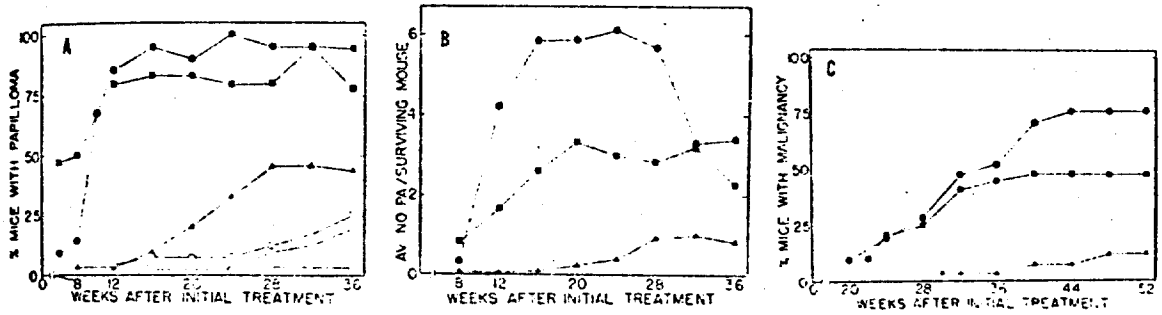



CHART 2.—The tumor incidence of mice treated with phenol or croton oil, with and without pretreatment with DMBA.
A. The percentage of surviving mice bearing one or more papillomas.
B. The average number of tumors per surviving mouse.
C. The percentage of the effectual total showing malignant tumors.

| Group | Symbol | Initial treatment | Secondary treatment* | Survivors† | Av. Wt.† (gm.) |
|---|---|---|---|---|---|
| 1 | No tumors | 75 µg. DMBA | Benzene | 28 | 37.0 |
| 2 | ▲ | " | 5% Phenol | 28 | 37.9 |
| 3 | ■ | " | 10% Phenol | 29 | 33.4 |
| 4 | ● | " | 0.5% Croton oil | 21 | 36.2 |
| 5 | △ | None | 5% Phenol | 30 | 38.9 |
| 6 | | " | 10% Phenol | 24 | 35.0 |
| 7 | | " | 0.5% Croton oil | 30 | 35.7 |

* The secondary treatments were given in quantities of 25 µl twice weekly for 51 weeks. There was a delay of 1 week between the initial and secondary treatments, and this delay was maintained also in Groups 5, 6, and 7.

† The number of surviving mice and the average weights are recorded for the 20th week, at which time the first malignant tumors appeared in Groups 3 and 4. Therefore, the number of survivors shown constitutes the effectual total for the calculation of incidence of malignant tumors shown in (C). The original number of mice was 30 for all groups except 22 in Group 4.

*The effect of the purity of phenol.*—The possibility was tested that the promoting activity of phenol might be attributable to a contaminant carried by the phenol. To do this, the promoting activity of a sample of U.S.P. grade phenol was compared with a purified preparation made from it (see "Materials and Methods"). Female mice, 2–3 months of age and obtained from Arthur Sutter, were used. The mice were treated as follows: Group 1 (nineteen mice) was given a single application of 75 μg. of DMBA in 25 μl. of benzene, and after an interval of 1 week 25 μl. of benzene was applied to the same area of the back

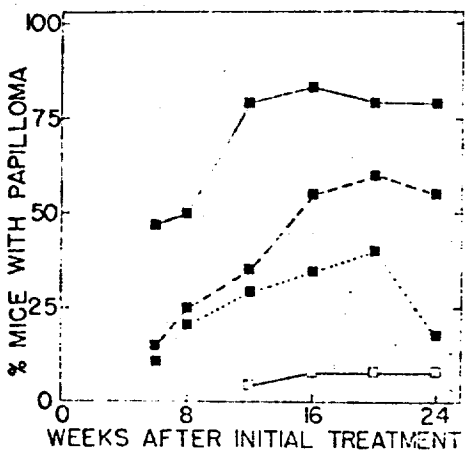



CHART 3.—The effect of strain of mouse on the response to phenol treatment, with and without pretreatment with DMBA.

*Group 1:* ■ - - - - □ $CAF_1$ mice; DMBA once, followed by twice-weekly applications of 10 per cent phenol.

*Group 3:* ■ · · · · ■ C3H mice; the same treatment as for group 1. ■ —— ■ Holtzman mice; from Chart 2A, Group 3 given the same treatment as Group 1. □ —— □ Holtzman mice; from Chart 2A, Group 6, treated with 10 per cent phenol alone; no DMBA.

No papillomas appeared in Groups 2 and 4, treated with 10 per cent phenol alone; no DMBA.

twice a week for the duration of the experiment (solvent control group). Group 2 was treated similarly with DMBA, and this was followed after 1 week with twice-weekly applications of 25 μl. mouse of a 5 per cent solution of U.S.P. grade phenol in benzene. Group 3 was also treated similarly with DMBA followed by twice-weekly applications of a 5 per cent solution of the purified phenol.

There was no evidence that the nonpurified phenol was a more effective promoting agent than the purified preparation. For example, at 20 weeks no tumors had appeared in the control mice (Group 1), while the incidence of papillomas in Group 2 (U.S.P. phenol) was 37 per cent compared with 52 per cent in the group exposed to the purified phenol. The average number of papillomas per mouse was 0.7 in each group at 20 weeks and 0.88 compared with 0.90 at 26 weeks.

*The effect of strain of mice.*—The great variation in the tumor response of mice of different strains to repetitive percutaneous administration of a carcinogenic hydrocarbon is well established (12), but little is known about the efficacy of two-stage carcinogenesis (initiator followed by promoter) in various strains. The response to a single application of DMBA followed by phenol was determined in two strains (C3H and $CAF_1$, both obtained from Roscoe B. Jackson Laboratory), and the result was compared with that obtained in Holtzman mice. The groups were as follows: Group 1: twenty $CAF_1$ female mice treated once with 75 μg. of DMBA followed after 1 week by 1 drop per mouse of 10 per cent phenol twice a week for the duration of the experiment. Group 2: twenty $CAF_1$ female mice given multiple applications of phenol as in Group 1 but without the preliminary treatment with DMBA. Group 3: twenty female C3H mice treated the same as the $CAF_1$ mice of Group 1; both the initiator and promoter were given. Group 4: twenty C3H female mice treated the same as the $CAF_1$ mice of Group 2; only the promoting treatment was given.

The percentage of mice bearing one or more papillomas is given for the first 24 weeks in Chart 3. The data for Holtzman mice (taken from Chart 2), treated with (*a*) DMBA followed by phenol both in the same amount given to the C3H and $CAF_1$ mice, and (*b*) phenol alone, are included for comparison. In the Holtzman mice the tumor incidence rose rapidly and plateaued at about 80 per cent of the mice after the 12th week. Tumors appeared more slowly in the two inbred strains reaching a maximum of 60 per cent in the $CAF_1$ mice and 42 per cent in the C3H mice at the 20th week. No tumors resulted in either of the inbred strains treated with phenol alone in contrast to almost 10 per cent tumor incidence in the Holtzman mice so treated. The average number of papillomas per mouse (Table 1) also indicated that Holtzman mice were the most responsive to skin applications of DMBA and phenol, while $CAF_1$ and C3H were less responsive. The maximum average number of papillomas obtained per mouse was 3.4, 1.4, and 0.7, respectively.

Epithelial outgrowths that did not resemble typical papillomas were observed in nearly all the $CAF_1$ and C3H mice treated with phenol alone. These growths were very small but distinct (about 0.5 mm. in diameter and protruding up to 1 mm.). They were found on the sides of the mice near the periphery of the denuded area over which the

phenol applications extended. A typical section is shown in Figure 4.

The applications of phenol to the $CAF_1$ and C3H mice were continued for 1 year, and the appearance of carcinomas was recorded. None appeared in the mice treated with phenol alone, but treatment with a single application of DMBA followed by phenol caused carcinomas to appear in both strains beginning after 6 months of treatment. At 1 year the incidence of carcinoma (based on effectual total) was 21 per cent in the $CAF_1$ mice and 29 per cent in the C3H mice.

*The promoting activity of compounds related to phenol.*—In all, fourteen separate experiments were done in which pertinent compounds were tested. Each group within an experiment consisted of from nineteen to 36 animals picked at random from a common pool of female Sutter mice, 2–3 months of age. The conditions of each of these experiments are given in Table 3 with respect to initiation, the solvent for and concentration of the agent tested as a promoter, and the elapsed time after initiation at which the data were taken (it is clear from Charts 2A and 2B that the choice of time is important). The compounds tested were obtained commercially (mostly Aldrich Chemical Co., Inc., Milwaukee, Wis., and Distillation Products Industries, Rochester, N.Y.) and were used as obtained. One drop (adjusted to approximately 25 μl.) of the test solution was applied to the back of each mouse twice weekly for the duration of the experiment. In the case of two agents (noted in the table), 2 drops were applied 6 times weekly for the duration of the experiment. In two experiments no initiator was used, time being measured from the first application of test substance. After it became clear that promoting activity of phenol derivatives followed certain patterns so that more refined methods of assay were needed, the test agents were applied at levels equimolar to a control solution of phenol.

The response was measured in three ways. First, the percentage of surviving mice bearing one or more papilloma was ascertained. Second, the total number of papillomas on all the surviving mice was counted and divided by the number of surviving mice to give the average number of papillomas per mouse. Finally, the number of mice bearing malignant tumors was determined; the latter measure was presented as the percentage of the animals surviving on the day chosen for presentation of the data, thus partially correcting for large numbers of nontumor deaths caused by toxic levels of phenols. These data on tumor response, together with the original and surviving number of mice in each group, are presented in Table 2, and a summary of these data, showing the effect of position and nature of substituent on the estimated comparative promoting activity, is provided in Table 3.

Substitution with a single halogen atom or methyl group in any one of the three possible positions had little effect on promoting activity; the resulting compounds were as potent as phenol or even more potent in the case of 4-fluorophenol. Of the six possible dimethyl phenols, all but the 2,3-dimethylphenol were tested. The 2,4-, 2,5-, 3,4-, and 3,5-dimethylphenols (exp. 10) were as potent as phenol, but the 2,6- isomer was only weakly effective as a promoting agent. Of the trisubstituted phenols, the 2,4,6-tribromo- and trichlorophenols as well as 2,3,5-trimethylphenol were inactive, while 2,4,5-trichlorophenol retained activity. No tumors developed in mice treated with pentachlorophenol. In this series of compounds, irritation and hair loss paralleled promoting activity. For example, mice treated with 2,6-dimethylphenol showed minimal effects of treatment (Fig. 5), while each of the other four dimethyl isomers caused skin damage, fur loss, and tumor formation (illustrated by 3,4-dimethylphenol, Fig. 6). Similarly, the inactive tri- and penta-substituted phenols caused no fur loss or wounding.

TABLE 1

THE EFFECT OF STRAIN ON THE NUMBER OF PAPILLOMAS IN MICE*

Each mouse was given an initial dose of 75 μg. of dimethylbenzanthracene (DMBA) followed by phenol.

| Strain | Time (weeks) | | | | |
|---|---|---|---|---|---|
| | 12 | 16 | 18 | 20 | 24 |
| Holtzman | 1.7 | 2.6 | | 3.4 | 3.0 |
| $CAF_1$ | 0.7 | | 1.4 | | 1.4 |
| C3H | 0.5 | | 0.7 | | 0.2 |

* The number of papillomas per mouse was calculated by dividing the total number of papillomas by the number of surviving mice at the time periods stated.

The promoting activity of mono-alkyl-substituted phenols was tested, with the length, saturation, and branching of the alkyl group being varied. From Table 2 it is seen that most of these compounds were tested simultaneously in Experiment 12 at levels equimolar to phenol. From the summary in Table 3, it is apparent that an alkyl group with 1 carbon (methyl) in either the 2 or 4 position did not alter the promoting action of the phenol molecule, but an alkyl group of 2, 3, 4, or 5 carbons in either the 2 or 4 position resulted in

## TABLE 2

### THE INCIDENCE OF TUMORS IN MICE TREATED WITH DERIVATIVES OF PHENOL AND CERTAIN OTHER AGENTS

The substances are presented by experiments consisting of groups of mice from a common pool that were tested simultaneously. The conditions of initiation, the solvent for the promoting agent, and the time after initiation at which the data were taken* are shown for each experiment.

| Promoter (applied twice weekly) | No. mice (survivors/original) | Av. pa.† per survivor | Per cent survivors with pa. | Per cent survivors with ca.‡ |
|---|---|---|---|---|
| Exp. 1.—Initiator: 0.3% DMBA in acetone. Promoter: in acetone. Data: at 18 wk. | | | | |
| None | 25/25 | 0 | 0 | 0 |
| 0.5% croton oil | 21/25 | 1.05 | 52 | 0 |
| 3.0% 1,4-naphthoquinone | 19/20 | 0.11 | 10 | 0 |
| 3.0% 2-methyl-1,4-naphthoquinone | 20/20 | 0 | 0 | 0 |
| 20% bromobenzene | 20/20 | 0 | 0 | 0 |
| 20% 4-bromophenol | 19/20 | 1.26 | 63 | 0 |
| 100% styrene (no solvent) | 19/20 | 0.19 | 16 | 0 |
| Exp. 2.—Initiator: 0.3% DMBA in benzene. Promoter: in benzene. Data: at 18 wk. | | | | |
| None (benzene control) | 16/17 | 0 | 0 | 0 |
| 0.5% croton oil | 28/31 | 1.93 | 61 | 0 (11% at 48 wk.) |
| 5.0% croton oil (solvent: mineral oil) | 21/30 | 2.14 | 57 | 0 (24% at 48 wk.) |
| 20% 4-fluorophenol | 11/30 | 2.64 | 91 | 18 (36% at 25 wk.) |
| 20% 4-iodophenol | 31/32 | 0.47 | 35 | 3 (6% at 28 wk.) |
| Exp. 3.—Initiator: 0.3% DMBA in acetone. Promoter: in acetone. Data: at 12 wk. | | | | |
| 20% phenol | 21/24 | | 58 | 5 |
| 20% 2,4-dinitrophenol | 36/36 | | 0 | 0 |
| 20% 2,4,6-tribromophenol | 33/36 | | 3 | 0 |
| 20% phenylacetate | 36/36 | | 6 | 0 |
| 20% methylphenylether | 34/36 | | 9 | 3 |
| 20% 2-naphthol | 33/36 | | 3 | 0 |
| 20% 1,4-dihydroxybenzene | 24/24 | | 0 | 0 |
| Exp. 4.—Initiator: 0.3% DMBA in acetone. Promoter: in benzene. Data: at 12 wk. | | | | |
| None (benzene control) | 12/12 | 0 | 0 | 0 |
| 20% fluorobenzene | 20/24 | 0 | 0 | 0 |
| 20% phenol | 22/27 | 1.50 | 64 | 0 |
| 20% 2-methylphenol | 17/27 | 1.35 | 59 | 0 |
| 20% 3-methylphenol | 14/29 | 0.93 | 50 | 0 |
| 20% 4-methylphenol | 20/28 | 0.55 | 35 | 0 |
| 20% 1,3-dihydroxybenzene (acetone)§ | 24/27 | 0.29 | 17 | 0 |
| Exp. 5.—Initiator: 0.3% DMBA in benzene. Promoter: in benzene. Data: at 15 wk. | | | | |
| None (control) | 16/20 | 0.13 | 13 | 0 (6% at 53 wk.) |
| 0.5% croton oil | 26/30 | 1.38 | 50 | 4 (8% at 53 wk.) |
| 20% 2,4-dimethylphenol | 28/30 | 1.21 | 50 | 11 (18% at 23 wk.) |
| 20% 2,6-dimethylphenol | 27/30 | 0.44 | 30 | 4 (11% at 23 wk.) |
| 20% 3,4-dimethylphenol | 21/30 | 2.66 | 95 | 0 (14% at 23 wk.) |
| 20% 3,5-dimethylphenol | 20/30 | 0.90 | 40 | 0 (5% at 23 wk.) |
| 15% 1,2-dihydroxybenzene | 22/30 | 0.14 | 14 | 0 |
| 20% 1,2,3-trihydroxybenzene | 28/30 | 0.04 | 4 | 0 |

* In some experiments, the data shown were taken from the final week. In others, promotion was continued beyond the time shown in order to allow carcinomas to develop, and, in these cases, the time chosen for presentation of the data was determined by the state of papilloma response. In each case, treatment with the promoting agent was begun 1 week after the single application of initiator and was continued twice each week until the experiment was discontinued.

† Abbreviations used include pa. for typical papilloma and ca. for epithelial carcinoma and, in rare cases, fibrosarcoma. In the first several experiments, all malignant lesions were verified by microscopic examination, while in later experiments frequent microscopic checks were made to verify the gross examinations.

‡ The incidence of carcinomas was calculated on the basis of the number of mice alive in each group on the date chosen for presentation of the data; this is not the usual definition for effectual total.

§ Because the agent was not sufficiently soluble in the solvent used in this experiment, sufficient acetone or alcohol was mixed with the specified solvent to effect solution.

TABLE 2—*Continued*

| Promoter (applied twice weekly) | No. mice (survivors/original) | Av. pa.† per survivor | Per cent survivors with pa. | Per cent survivors with ca.‡ |
|---|---|---|---|---|
| Exp. 6.—Initiator: 0.3% DMBA in benzene. Promoter: in benzene. Data: at 15 wk. | | | | |
| None | 15/20 | 0.07 | 7 | 0 |
| 0.5% croton oil | 14/20 | 0.64 | 29 | 0 |
| 20% 2-chlorophenol | 31/35 | 1.48 | 61 | 10 |
| 20% 3-chlorophenol | 21/33 | 1.38 | 67 | 0 |
| 20% 2,4-dichlorophenol | 27/33 | 1.07 | 48 | 11 |
| 20% 2,4,6-trichlorophenol | 26/29 | 0 | 0 | 0 |
| 20% pentachlorophenol | 29/35 | 0.04 | 4 | 0 |
| 20% 3-*tert*-butyl-4-hydroxyanisole | 33/34 | 0 | 0 | 0 |
| 20% 3,5-di-*tert*-butyl-4-hydroxytoluene | 28/33 | 0 | 0 | 0 |
| Exp. 7.—Initiator: 0.3% DMBA in benzene. Promoter: in benzene. Data: at 21 wk. | | | | |
| None (benzene control) | 20/23 | 0 | 0 | 0 |
| 2% croton oil | 22/24 | 4.00 | 91 | 0 |
| 20% 2-naphthol (solvent: ethanol) | 21/24 | 0 | 0 | 0 |
| 15% 1,4-naphthoquinone | 21/24 | 0.48 | 38 | 5 (10% at 34 wk.) |
| 20% 2-methyl-1,4-naphthoquinone | 20/23 | 0.15 | 15 | 5 |
| 20% 2-2′-methylenebis(3,4,6-trichlorophenol) | 20/23 | 0.10 | 10 | 5 |
| 20% 2-hexyl-4-chlorophenol | 19/23 | 0.84 | 58 | 0 (5% at 34 wk.) |
| 20% 2-benzyl-4-chlorophenol | 23/23 | 0 | 0 | 0 (4% at 34 wk.) |
| 15% bis(diethylthiocarbamoyl)disulfide (solvent: acetone) | 23/24 | 0 | 0 | 0 |
| 20% dodecylbenzene | 17/24 | 0 | 0 | 0 |
| 20% squalene | 17/23 | 0 | 0 | 0 |
| Exp. 8.—Initiator: 0.3% DMBA in benzene, Groups 1–4; none, Groups 5–9. Promoter: in benzene. Data: at 24 wk. | | | | |
| None (benzene control) | 27/32 | 0.15 | 11 | 0 |
| 5% phenol | 27/33 | 1.67 | 74 | 4 (26% at 40 wk.) |
| 10% phenol | 15/33 | 3.94 | 100 | 33 (93% at 39 wk.) |
| 20% phenol | 10/33 | 3.20 | 100 | 20 (70% at 38 wk.) |
| 5% phenol | 25/33 | 1.16 | 56 | 4 (12% at 40 wk.) |
| 10% phenol | 19/33 | 2.68 | 95 | 37 (68% at 39 wk.) |
| 20% phenol | 20/33 | 2.25 | 90 | 15 (65% at 40 wk.) |
| 20% 2,4-dichlorophenol | 16/23 | 1.62 | 75 | 6 (62% at 39 wk.) |
| 20% 2,4-dimethylphenol | 19/24 | 1.42 | 63 | 5 (42% at 39 wk.) |
| Exp. 9.—Initiator: none. Promoter: in dioxane. Data: at 12 wk. | | | | |
| 20% phenol | 16/30 | 0.94 | 63 | 0 |
| 20% 2-chlorophenol | 28/30 | 0.64 | 46 | 0 |
| 20% 2-hydroxybenzaldehyde | 31/31 | 0 | 0 | 0 |
| 20% 2-hydroxybenzoic acid | 31/31 | 0 | 0 | 0 |
| 20% 2-nitrophenol | 30/31 | 0 | 0 | 0 |
| 20% 4-nitrophenol | 30/31 | 0 | 0 | 0 |
| 20% aniline | 30/31 | 0 | 0 | 0 |
| 20% *p*-toluidine | 27/32 | 0 | 0 | 0 |
| Exp. 10.—Initiator: none. Promoter: in benzene. Data: at 20 wk. | | | | |
| 10% phenol | 24/30 | 0.62 | 33 | 13 (29% at 28 wk.) |
| 10% 2,4-dimethylphenol | 26/29 | 0.66 | 31 | 0 (12% at 28 wk.) |
| 10% 2,5-dimethylphenol | 25/30 | 0.40 | 24 | 0 (8% at 28 wk.) |
| 10% 2,6-dimethylphenol | 26/30 | 0.15 | 8 | 0 |
| 10% 3,4-dimethylphenol | 28/29 | 0.71 | 50 | 4 (14% at 28 wk.) |
| 10% 3,5-dimethylphenol | 22/30 | 0.91 | 55 | 5 (14% at 28 wk.) |

† This is not the same experiment on levels of phenol shown in Figure 2.

TABLE 2—*Continued*

| Promoter (applied twice weekly) | No. mice (survivors/original) | Av. pa.† per survivor | Per cent survivors with pa. | Per cent survivors with Ca.‡ |
|---|---|---|---|---|
| Exp. 10—*Continued* | | | | |
| 13.1% 4-methoxyphenol‖ | 25/30 | 0.04 | 4 | 0 |
| 17.2% 4-*tert*-amylphenol‖ | 22/30 | 0 | 0 | 0 |
| 18.1% 2-phenylphenol‖ | 24/30 | 0.04 | 4 | 0 |
| 5% *p*-phenylenediamine** | 19/30 | 0 | 0 | 0 |
| Exp. 11.—Initiator: 0.3% DMBA in acetone. Promoter: in acetone. Data: at 16 wk. | | | | |
| None (acetone control) | 18/20 | 0 | 0 | 0 |
| 10% phenol | 19/20 | 0.63 | 32 | 0 (5% at 30 wk.) |
| 10% 4,4′-dihydroxybiphenyl†† (ethanol)§ | 20/20 | 0 | 0 | 0 |
| 16% 4-*tert*-butylphenol‖†† | 17/19 | 0.06 | 6 | 0 |
| 16% 5,6,7,8-tetrahydro-2-naphthol‖ | 16/20 | 1.00 | 63 | 6 (25% at 30 wk.) |
| 21% 2,4,5-trichlorophenol‖ | 19/20 | 0.95 | 42 | 0 (5% at 30 wk.) |
| 14% 2,3,5-trimethylphenol‖†† | 20/20 | 0.05 | 5 | 0 |
| 14% 2-allylphenol‖ | 17/19 | 1.24 | 35 | 0 (24% at 30 wk.) |
| 14% 4-hydroxybenzoic acid‖†† | 19/19 | 0.05 | 5 | 0 |
| Exp. 12.—Initiator: 0.3% DMBA in benzene. Promoter: in benzene. Data: at 12 wk. | | | | |
| None (benzene control) | 18/20 | 0 | 0 | 0 |
| 10% phenol | 16/20 | 2.62 | 88 | 0 |
| 13% 2-ethylphenol‖ | 20/20 | 0.60 | 55 | 0 |
| 14% 2-allylphenol‖ | 20/20 | 0.75 | 55 | 0 |
| 19% 2,6-diallylphenol‖ | 20/20 | 0.35 | 25 | 0 |
| 14% 2-propenylphenol‖ | 19/20 | 0.37 | 26 | 0 |
| 16% 2-isopropyl-4-methylphenol‖ | 16/19 | 0.19 | 19 | 6 |
| 16% 2-crotonylphenol‖ | 19/20 | 0.47 | 26 | 0 |
| 16% 2-*n*-butylphenol‖ | 19/20 | 1.26 | 58 | 0 |
| 17% 2-*sec*-amylphenol‖ | 19/20 | 0.79 | 42 | 0 |
| 16% 4-*n*-butylphenol‖ | 19/20 | 0.21 | 16 | 0 |
| 17% 4-*n*-amylphenol‖ | 18/20 | 0.22 | 11 | 0 |
| 17% 4-*sec*-amylphenol‖ | 15/20 | 0.73 | 40 | 0 |
| 17% 4-*tert*-amylphenol‖ | 14/18 | 0 | 0 | 0 |
| 22% 4-(1,1,3,3-tetramethylbutyl)phenol‖ | 18/20 | 0.28 | 11 | 0 |
| Exp. 13.—Initiator: 0.3% DMBA in benzene. Promoter: in benzene. Data: at 20 wk. | | | | |
| None (benzene control) | 18/20 | 0 | 0 | 0 |
| 5% phenol | 13/19 | 0.46 | 31 | 8 |
| 10% phenol | 12/20 | 2.08 | 83 | 8 |
| 5.7% 3-methylphenol‡‡ | 17/20 | 0.24 | 24 | 0 |
| 5.7% 4-methylphenol‡‡ | 14/20 | 0.36 | 29 | 0 |
| 7.9% 5,6,7,8-tetrahydro-2-naphthol‡‡ | 17/20 | 0 | 0 | 0 |
| 20% 4-chlorothiophenol | 13/20 | 0.23 | 15 | 0 |
| 20% biphenyl (at 16 wk.) | 16/20 | 0 | 0 | 0 |
| 1.5% 2,3-dichloro-1,4-naphthoquinone (sat., 2 drops/mouse, 6/week) | 17/20 | 0.18 | 18 | 0 |
| 100% turpentine (no solvent, 2 drops/mouse, 6/week) | 18/20 | 0.28 | 17 | 0 |
| Exp. 14.—Initiator: 0.4% DMBA in acetone. Promoter: 30% ethanol in acetone. Data: at 14 wk. | | | | |
| None (ethanol-acetone control) | 20/20 | 0 | 0 | 0 |
| 9.4% (1 M) phenol | 19/20 | 0.26 | 16 | 0 |
| 14.8% (1 M) 5,6,7,8-tetrahydro-2-naphthol | 19/20 | 0.79 | 53 | 0 |
| 16.4% (1 M) tetralin-*p*-quinol | 19/20 | 0 | 0 | 0 |

‖ The concentration of these compounds was equimolar to 10% phenol.

** The *p*-phenylenediamine was freshly prepared in acetone for each application.

†† These four groups were discontinued at 12 weeks; hence, the data are for the 12th week rather than the 16th week.

‡‡ The concentration of these compounds was equimolar to 5% phenol.

diminished activity with the exception of 2-*n*-butylphenol. In fact, no activity was shown by the 4-*tert*-butyl- and 4-*tert*-amylphenols. Unsaturation in the alkyl substituent (the allyl-, propenyl-, and crotenyl-derivatives) had no clear effect on activity.

The effect of several other types of substituents is also summarized in Table 3. Of the three possible dihydroxybenzenes known as catechol, resorcinol, and hydroquinone, only resorcinol showed activity, and this was probably questionable. Pyrogallol (1,2,3-trihydroxybenzene) was inactive. The introduction of nitro groups into the ring completely destroyed the promoting effect of phenol. The presence of either a carbonyl, a carboxyl, or a methoxy group attached to the ring also destroyed activity. Furthermore, the free -OH group of phenol was required to retain promoting activity; both the methyl ether of phenol (anisole) and the acyl derivative (phenylacetate) were unable to elicit many tumors.

In studies on the metabolism of 5,6,7,8-tetrahydro-2-naphthol (tetralol), Hecker and Mueller (10) demonstrated a protein-bound derivative of this compound as well as a new metabolite identified as 5,6,7,8-tetrahydro-2-naphthylhydroperoxide (tetralin-*p*-quinol). These findings suggested the evaluation of the compounds as promoting agents.[3]

[3] The authors are indebted to Dr. Gerald C. Mueller for suggesting the importance of testing these compounds for promoting action.

When tested at equimolar levels, 2-naphthol was only moderately active, while tetralol was at least as effective as phenol itself and the quinol derivative was inactive.

Although not closely related to phenolic compounds, 1,4-naphthoquinone, 2-methyl-1,4-naphthoquinone, and 2,3-dichloro-1,4-naphthoquinone were tested. The first two showed low activity in two experiments, and the latter compound showed questionable effect in a single test. These results are in agreement with those of Kline and Rusch (11) and of Gwynn and Salaman (9).

Activity was less clearly demonstrated in biphenyl derivatives than in compounds based on condensed (naphthalene) ring systems. Thus, the effect of 2-phenylphenol was doubtful, while no response to 4,4′-dihydroxybiphenyl was evident.

Low activity was possessed by 2-benzyl-4-chlorophenol, while the highly substituted 2,2′-methylenebis(3,4,6-trichlorophenol) was inactive. Both of these were tested at levels (5 mg/mouse, twice each week) that caused considerable skin irritation.

It was of interest that 4-chlorobenzenethiol showed low activity. Neither fluorobenzene nor *p*-phenylenediamine, a known skin irritant, promoted the appearance of papillomas.

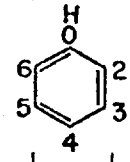
## DISCUSSION

The capacity of phenol to elicit epithelial tumors in mice in areas previously treated with sub-

TABLE 3

THE ESTIMATED COMPARATIVE PROMOTING ACTIVITY* OF SUBSTITUTED PHENOLS SHOWING THE EFFECT OF THE NATURE OF THE SUBSTITUENT AND OF THE POSITION SUBSTITUTED

| Substituent | Position | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 2 | 3 | 4 | 2,3 | 2,4 | 2,5 | 2,6 | 3,4 | 3,5 | 2,3,5 | 2,4,5 | 2,4,6 | 2,3,4,5,6 |
| Halogen† | ++ | ++ | ++ | | ++ | | | | | | ++ | − | − |
| $CH_3$ | ++ | ++ | ++ | | ++ | ++ | ± | ++ | ++ | − | | | |
| $C_2H_5$ | + | | | | | | | | | | | | |
| $C_3H_7$ allyl | + | | | | | | + | | | | | | |
| propenyl | + | | | | | | | | | | | | |
| $C_4H_9$ normal | ++ | | + | | | | | | | | | | |
| crotenyl | + | | | | | | | | | | | | |
| tert. | | | − | | | | | | | | | | |
| $C_5H_{11}$ sec. | + | | + | | | | | | | | | | |
| tert. | | | − | | | | | | | | | | |
| $C_6H_4$ phenyl | − | | | | | | | | | | | | |
| OH | − | ± | − | − | | | | | | | | | |
| NO | − | | − | | − | | | | | | | | |
| CHO | − | | | | | | | | | | | | |
| COOH | − | | | | | | | | | | | | |
| $CH_3O$ | | | − | | | | | | | | | | |




* The compounds are rated as follows: ++, same order of activity as phenol; +, low activity; ±, questionable activity; −, no activity.

† Each of the four halogens was substituted in 1 or more of the positions noted. Because no difference in the relative effectiveness of the various halogens was noted (especially considering the great variation in molecular weight), results with individual halogens are not presented in this summary.

carcinogenic amounts of DMBA has been demonstrated many times in mice of several sources and strains. The same schedule of phenol applied to the skin of similar mice not previously treated with DMBA was much less effective or, in the case of C3H and $CAF_1$ strains, caused no papillomas at all. The results are entirely analogous to those obtained with croton oil; the response to a small amount of DMBA followed by phenol is much greater than the response to several times as much of either agent alone. These facts suggest that promoting action as defined by Friedwald and Rous (7) and by Berenblum and Shubik (1) is an attribute of phenol and many phenolic compounds; but, in addition, it is clear that phenol as well as croton oil (3, 13, 14), when adequately tested in susceptible mice, is carcinogenic in itself.

The possibility of contamination of phenol with known or unknown carcinogens exists because the usual source of phenol, or of the benzene for synthetic phenol, is coal tar. Therefore, a careful laboratory purification, designed to extract the neutral hydrocarbons from phenol followed by a repeated distillation from zinc dust, was made. Because no evidence was found of loss of activity after purification, both promoting and weak carcinogenic activities are attributed to phenol itself. This conclusion gains further support from the observations on the activity of the phenol derivatives. In tests on over 50 different compounds, the activity followed logical variations in structure rather than appearing randomly among derivatives, as might be expected of a contaminant.

The tumor response of groups of mice exposed to graded amounts of phenol showed that a maximal response was reached at the level of 2.5 mg. of phenol per mouse (a 10 per cent solution) twice a week. A lesser response was obtained at the same schedule of 1.25 mg. of phenol, while 5.0 mg. of phenol per application (a 20 per cent solution) caused a number of deaths as a result of systemic toxicity (Table 2). In addition, the development of papillomas was prevented by the corrosive effects on the skin at the highest level. After equivalent initiating action, twice-weekly applications of 2,500 μg. each of phenol (10 per cent) caused nearly as many papillomas as an equal number of applications of only 125 μg. each of croton oil (0.5 per cent). Similarly, all chemically defined promoting agents so far reported, such as iodoacetic acid (9) and certain surface-active agents (18), are much less effective per unit weight than croton oil. This difference is even greater in terms of the content of the biologically active components in croton oil (8, 19).

Certain structural requirements for the retention of promoting activity by phenolic compounds have been demonstrated. A need was found for at least one unsubstituted position ortho to the phenolic group. Although monomethyl-substituted phenols retained full activity, the presence of larger aliphatic groups depressed activity, especially if highly branched. The presence of substituents on ring carbons such as nitro, carbonyl, and carboxyl groups, or a second phenolic group, destroyed promoting activity. No simple interpretation of these facts is possible, but it is probable that an active ortho position must be available to react with a tissue constituent.

The known biological effects of phenol and its derivatives (20) have been studied for clues to the mechanism of the promoting action. These include bactericidal and fungicidal action, enzyme and metabolic effects, mitotic poisoning in the sea urchin egg (6), and various toxicologic manifestations on the circulatory and nervous systems, as well as general tissue destruction (corrosive action). No correlation has been found between the promoting activity of the large number of phenol derivatives that were tested and the relative effectiveness of the same derivatives for known biological effects. The capacity of phenolic compounds to act as anti-oxidants did not correlate with promoting activity. Phenol is known to rupture hydrogen bonds and to coagulate or, at high concentrations, to dissolve protein, aiding in the deproteinization of nucleic acid (17). Because the activity of phenol derivatives for these functions is not known, no correlations were possible. However, a quantitative study of the binding to skin proteins of $C^{14}$-labeled phenol showed that only a very small proportion of the applied radioactivity was firmly bound to protein.[4]

Based on gross observations, the destruction of sebaceous glands, wounding, and fur loss were not essential attributes of promoting agents. For example, phenol at the 5 per cent level (Fig. 2), croton oil dissolved in mineral oil, and lower levels of certain surface-active agents have been found to promote tumors with no gross signs of tissue damage or hair loss. However, at higher and more effective levels, promoting agents generally show destructive effects on skin tissues. Conversely, a number of compounds have been observed to cause extensive skin damage without evidence of promoting activity (e.g., styrene). However,

[4] F. J. C. Roe, B. Booth, and R. K. Boutwell, unpublished data.

knowledge of the biochemical changes essential to carcinogenesis should resolve the confusion on the role of cell division (15), irritant action (16), sebaceous gland destruction (2), and fur loss in carcinogenesis. It is possible that the macroscopic effects may be extrapolated to the microscopic picture; even in amounts insufficient to produce gross damage, the agents effective in carcinogenesis cause a number of cells to die. It is suggested that the increased mitotic frequency which has been observed after treatment with carcinogens and promoting agents (15) is the result of repair processes and is not an essential part of the carcinogenic reaction. Rather, it may be a manifestation of a high frequency of lethal effects on cells of these agents in proportion to the more rare changes leading to neoplasia.

The hazard of phenolic compounds to man must now be considered. Contacts with phenols range from heavy industrial contacts through more intermittent contact with disinfectants, preservatives, anti-oxidants and the like to atmospheric pollution. (The Battelle Memorial Institute has reported that for every ton of rubbish burned, more than 8 pounds of the emissions are phenols.[5]) Although chronic phenol poisoning resulting from industrial contact has been observed, we are aware of no association between exposure to phenol and the incidence of human cancer. It is pertinent that small amounts of phenolic compounds are formed in the animal body, at least in part by bacterial metabolism in the intestine. The results with mice suggest that even in susceptible animals tumors result only after continued exposure to relatively large quantities of the reagent. Further studies on the relationship between the amount and frequency of exposure to phenol and the tumor response are in progress in order to gain more knowledge about the practical hazards and mechanism of action of phenol in particular, and of promoting agents in general.

## SUMMARY

1. Phenol, dissolved in benzene or acetone, was applied repeatedly to the skin of the backs of mice either alone or following a single application of dimethylbenzanthracene (DMBA).

2. Papillomas appeared rapidly and in large numbers after treatment with DMBA followed by repeated applications of a 10 per cent solution of phenol; carcinomas appeared more slowly. Phenol alone was capable of eliciting tumors.

3. The capacity of over 50 different compounds related to phenol to promote the appearance of tumors after a single application of DMBA was also determined.

4. The response was dependent on the quantity of phenol applied, the susceptibility of the mice used, and the structure of the phenolic compound. Evidence that the response was due to phenol and not to a contaminant was presented.

[5] This report appeared in *Chemical and Engineering News* for Dec. 26, 1955, p. 5578.

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Fig. 1.—A section of a fibrosarcoma that developed on the back of a mouse treated with phenol alone. ×750.

Fig. 2.—Typical mice 36 weeks after a single application of 75 μg. of DMBA followed after 1 week by twice-weekly applications of 5 per cent phenol in benzene. Note the normal appearance of the hair coat.

Fig. 3.—Typical mice 36 weeks after a single applica 75 μg. of DMBA followed after 1 week by twice-week plications of 10 per cent phenol in benzene. At this time papillomas had regressed (Chart 2B), and nearly 50 pe of the mice bore malignant neoplasms (Chart 3). Note t tensive skin damage caused by 10 per cent phenol.

# THE EFFECT OF THE LEVEL OF DIETARY FAT ON THE TOXICITY OF PHENOLIC ANTIOXIDANTS

by W. D. BROWN,[1] A. R. JOHNSON AND M. W. O'HALLORAN[2]

(From the Commonwealth Antioxidant Research Project, University of Adelaide).

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## SUMMARY.

A study was made of the effect of two antioxidants, butylated hydroxy anisole (BHA) and butylated hydroxy toluene (BHT), with variation in the type and quantity of dietary fat, on growth, food consumption, reproduction, mortality, organ weights and post-mortem pathology of the rat.

The normal cellular behaviour of experimental animals fed diets containing one hundred times the amount of the antioxidant normally expected to be present in human foods (normal testing dose, NTD), is the conventional yardstick of non-toxicity.

The NTD of BHA produced no apparent changes in any of the parameters measured. When BHT was fed at a dietary level of 0·1 p.c. (the NTD) in conjunction with a 20 p.c. lard supplement, it significantly reduced the initial growth rate and mature weight of male rats. No such significant effect was noted in female rats or in male rats with a 10 p.c. lard supplement. A paired feeding experiment showed that this inhibition of growth was a direct toxic effect of BHT and could not be explained by a reduction in the palatability of the diet.

At the NTD, BHT produced a significant increase in the weight of the liver relative to the body weight. The mean absolute weight of the liver was also increased.

A significant loss of hair on the top of the head occurred in animals whose diet contained the NTD of BHT. This effect was only noted in animals under conditions of increased stress and was enhanced by an increase in the lard content of the diet. Anophthalmia occurred in 10 p.c. of the litters born of parents fed BHT.

Even when present in the diet at a concentration of 0·5 p.c., BHA and BHT had no effect on one reproductive cycle of the rat, or the histology of the spleen, kidney, liver, testes or skin, or the ratio to total body weight of the weight of the heart, spleen and kidney.

If the conventional NTD be accepted, BHA fulfilled the requirements of non-toxicity. BHT, at the NTD, on the other hand, produced deleterious deviations from normal cellular behaviour which cast doubt on the advisability of permitting this antioxidant in foodstuffs for human consumption.

## INTRODUCTION.

Butylated hydroxy anisole (BHA) and butylated hydroxy toluene (BHT) are now extensively used as antioxidants in fats for human consumption (Kray-

[1] Present address: Kanematsu Memorial Institute for Pathology, Sydney Hospital, Sydney, Australia.

[2] Present address: Nicholas Institute of Medical and Veterinary Research, Burnham Beeches, Sherbrook, Victoria, Australia.

bill *et al.*, 1949; Dugan *et al.*, 1954). The normal level of usage is 0·01-0·02 p.c. of the fat. Wilder and Kraybill (1948), Graham *et al.* (1954), Graham and Grice (1955), Deichmann *et al.* (1955) believe these antioxidants do not have toxic effects when fed to several species of animals at a concentration 100 times their normal level of usage in human diets. This safety factor of 100 is usually referred to as the normal testing dose (NTD) for the safety of a food additive, and is generally accepted as a prerequisite for the proof of non-toxicity of the material for use in human foods (Lehman *et al.*, 1951; Frazer, 1952; Johnson, 1959).

In the case of BHT, no reproductive studies were carried out in the above investigations; but Ames *et al.* (1956) and Draper *et al.* (1958) have shown that, so far as reproduction is concerned in the rat, BHT will not replace vitamin E, also an antioxidant, and that BHT at a dietary level of 0·3 p.c. does not interfere with reproduction.

The absorption of many lipids depends on the dietary content of triglyceride, and it seemed probable that the lipid antioxidants would be similarly affected. Consequently the effects of variation of the type and quantity of dietary fat on the toxicity of BHA and BHT were examined in the present investigation. Toxic effects were judged from growth, food consumption, reproduction, mortality, organ weights, and morbid pathology. The biochemical effects of the antioxidants have been reported elsewhere (Johnson *et al.*, 1958; Day *et al.*, 1959).

## Methods.

The animals used in these investigations were weanling (28 days) rats of the Norway Hooded or Albino strain whose parents had been maintained on a stock diet which consisted of rolled oats 300 gm., crushed wheat 300 gm., B mixture 300 gm., cooked beef mince 80 gm., water 200 ml., milk 80 ml., and "Potantol"[3] 0·5 ml. The B mixture contained pollard (shorts) 10 parts, wheat germ 10 parts, lactic casein 7 parts, full cream powdered milk 6 parts, sodium chloride 1·25 parts, and calcium carbonate 0·5 parts. Gravimetric determination of the petrol-ether soluble lipid contained in a hot alcohol-ether extract of dry samples of homogenized stock diet, showed that the diet contained 6·3 p.c. lipid on a wet weight basis or 8 p.c. dry weight. Table 1 shows the composition of the control and experimental diets used in this study.

The 0·001 p.c. (w/w) dietary level of antioxidant was the concentration when 5 p.c. (w/w) of the diet was fat stabilized with 0·02 p.c. antioxidant. The diet containing 0·1 p.c. antioxidant was the NTD. The diet containing 0·5 p.c. antioxidant was used in an attempt to induce pathological changes which might give information about the metabolic actions of the substance. The general methods for the care of the experimental animals, and the preparation of diets have been previously described (Johnson *et al.*, 1958; Day, *et al.*, 1959).

Each of the experiments was designed to permit a direct comparison of the various treatments on litter mates, and in the majority of cases a factorial design was used so that analysis of variance could be applied to the results.

[3] "Potantol" a commercial vitamin concentrate containing not less than 16,000 I.U. vitamin A and 2,000 I.U. vitamin D per ml.



TABLE 1.

*Fat, BHA, and BHT supplements to stock diet.*

| Antioxidant | Series No. and strain of rat | Duration of experiment | No. of animals* on each antioxidant level | Type and quantity of fat | Antioxidant levels in diets (p.c. dry weight) |
|---|---|---|---|---|---|
| BHA** | A Albino | 2 years | 26 | 10 p.c. hydrogenated coconut oil | 0·01, 0·1 |
| | B Norway Hooded | 8 months | 40 | 10 p.c. lard | 0·0, 0·001 0·1, 0·5 |
| | | | 40 | 10 p.c. lard heated | 0·0, 0·0004† 0·1, 0·5 |
| BHT*** | C Norway Hooded | 2 years | 24 | 10 p.c. hydrogenated coconut oil | 0·0, 0·1 |
| | D Norway Hooded | 6 months | 16 | 10 p.c. lard | 0·0, 0·1, 0·5 |
| | | | 16 | 20 p.c. lard | 0·0, 0·1, 0·5 |
| | E Norway Hooded | 7 weeks | 12 | 0 p.c. lard | (0·0-0·001‡) (0·1-0·0) (0·5-0·0) |
| | | | 12 | 10 p.c. lard | (0·0-0·001) (0·1-0·0) (0·5-0·0) |
| | | | 12 | 20 p.c. lard | (0·0-0·001) (0·1-0·0) (0·5-0·0) |

* Composed of equal numbers of male and female litter mates except series E when male rats were used.

** Tenox BHA, a commercial preparation containing approximately 85 p.c. 3-tert-butyl-4-hydroxy anisole and 15 p.c. 2-tert-butyl-4-hydroxy anisole.

*** Tenox BHT, 2:6-di-tert-butyl-p-cresol.

† See text.

‡ Indicates second litter mate of each diet pair, pair-fed against first.

In the long term experiments A and C the male and female litter mates were housed in pairs directly after weaning. When the female became pregnant she was removed to a separate breeding cage and allowed to rear the young until weaning. The original parents were then housed in pairs, the sexes being segregated. After 12 months on the dietary regimens, the same male and female litter mates were again mated; but unfortunately the fertile life span of the rats had been exceeded, and, in the majority of cases, no litters were produced. After two years the condition of both experimental and control survivors had so deteriorated that it was felt advisable to kill them for pathological examination.

In experimental series B the sexes were segregated, the animals being housed in groups of four. After three months on the dietary regimens one half of the animals were killed and used for organ weight, histopathological and fat stability studies. At six months male and female litter mates on the same diet were mated and the results of this mating recorded. At eight months the animals were killed, and organ weight and histopathological studies were made. To simulate commercial and domestic culinary practice, the lard used in one half of the control and experimental diets was heated to and held at 150° C. for 30 minutes in Kjeldahl flasks before being added to the stock diet.

In experimental series D the animals were caged in groups of four. Reproductive studies were carried out at the fourth month on the dietary regimens followed by killing at six months, and the determinations described above were carried out.

Experiment E was a paired feeding experiment in which the normal techniques of this method were applied. It was designed to assess the effect of BHT on growth and organ weights at a constant food intake. In this experiment the animals were caged singly.

In all experiments the animals were weighed weekly. The initial growth rate was calculated from the formulae $\frac{2W_4 + W_3 - W_1 - 2W_0}{10}$ ($W_4$ = weight at fourth week of experiment, etc.). The mature weight was taken as an average over seven weeks when the growth plateau was reached. The weights of the liver, kidney, heart, spleen and testes were related to the post-absorptive weight when killed. In all the above instances the resulting figures were transformed to logarithms for statistical analysis.

The liver, kidney, spleen, testes, aortae and skin of all animals of experiment D, and of a random selection of some animals from each dietary group in experiment B were examined histologically. The tissues were fixed in 10 p.c. formol-saline. Paraffin sections were cut at 5μ. The aortae were coiled longitudinally to include the whole length. The skin in the area between the ears was shaved and removed immediately after the animals were killed. This skin was stuck flat on a piece of cardboard, using an albumin-glycerine (1:1) mixture, without applying tension, and fixed in 10 p.c. formol-saline. Haematoxylin and Eosin (H and E) stains were used for general staining; toluidine blue (TB) and periodic acid Schiff (PAS) stains were used on sections from aortae and skin of all the animals in the D series. Control and test sections were stained in large groups to ensure comparable dye uptake.

## RESULTS.

### *Initial growth rate.*

An analysis of variance carried out on the results of experiment D is given in Table 2; all other results are given as mean values and the level of significant difference from the appropriate control quoted if applicable.

TABLE 2.

*Analysis of variance of the effect of BHT, lard and sex on the log initial growth rate of experimental series D rats.*

| Variation due to | d.f. | Sum of squares | Mean square | Variance ratio |
|---|---|---|---|---|
| Lards | 1 | 0·112273 | 0·112273 | 6·545 |
| Within Lards | 2 | 0·034307 | 0·017154 | |
| Between Litter Quads | 3 | 0·146580 | | |
| Sex | 1 | 0·315908 | 0·315908 | 169·025** |
| Sex × Lards | 1 | 0·003092 | 0·003092 | 1·654 |
| Treatments | 2 | 1·632966 | 0·816483 | 114·450*** |
| Sex × Treatments | 2 | 0·161189 | 0·080595 | 11·287** |
| Lard × Treatments | 2 | 0·085316 | 0·042658 | 5·98* |
| Sex × Lard × Treatments | 2 | 0·036174 | 0·018087 | 2·535 |
| Residual | 8 | 0·057074 | 0·007134 | |
| Between Cages | 23 | 2·442036 | | |
| Within Cages | 72 | 0·447549 | | |
| Total | 95 | 2·889585 | | |

*, **, *** significant at 5 p.c., 1 p.c., and 0·1 p.c. level respectively.



*The effect of BHA and BHT on the initial growth rate and mature weight of the rat.*

| Antioxidant | Experiment series | Type and quantity of fat | Sex | Level of antioxidants (p.c. dry weight of diet) 0 | | 0·0004 | | 0·001 | | 0·1 | | 0·5 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | IGR† | MW‡ | IGR | MW | IGR | MW | IGR | MW | IGR | MW |
| BHA | A | 10 p.c. coconut oil | Male | 29·7 | 376 | — | — | — | — | 32·3* | 377 | — | — |
| | | | Female | 20·6 | 249 | — | — | — | — | 22·9* | 235 | — | — |
| | B | 10 p.c. lard | Male | 22·6 | 305 | — | — | 25·8* | 306 | 23·8 | 308 | 20·2* | 298 |
| | | | Female | 14·7 | 183 | — | — | 15·1 | 182 | 13·8 | 186 | 12·0*** | 163* |
| | | 10 p.c. heated lard | Male | 24·0 | 319 | 24·3 | 336 | — | — | 23·7 | 325 | 22·7* | 287* |
| | | | Female | 14·1 | 173 | 14·2 | 176 | — | — | 14·6 | 180 | 13·6 | 174 |
| BHT | C | 10 p.c. coconut oil | Male | 34·7 | 442 | — | — | — | — | 33·2 | 427 | — | — |
| | | | Female | 22·6 | 241 | — | — | — | — | 21·5 | 239 | — | — |
| | D | 10 p.c. lard | Male | 23·3 | 266 | — | — | — | — | 23·1 | 272 | 12·6*** | 190*** |
| | | | Female | 16·7 | 165 | — | — | — | — | 15·3 | 162 | 11·0 | 144 |
| | | 20 p.c. lard | Male | 25·0 | 277 | — | — | — | — | 20·0* | 249* | 7·8*** | 190*** |
| | | | Female | 15·1 | 167 | — | — | — | — | 14·2 | 165 | 8·9*** | 152* |

† IGR = Initial growth rate.
‡ MW = Mature weight (gm.).
*, **, ***, Indicates that the logarithm of figure is significantly different from appropriate control at 0·05, 0·01 and 0·001 level of probability respectively.

The analysis shows that there is a significant difference between the sexes, and between treatments, and that there are two significant interactions: namely, Sex X Treatment and Lard X Treatment. The reasons for these interactions can be seen in Table 3; a diet containing the normal testing dose of BHT produced a significant reduction in the growth rate of male rats whose diet was supplemented with 20 p.c. lard. This effect was not noted in female rats nor in male and female rats whose diet contained 0·1 p.c. BHT with a 10 p.c. lard or coconut oil supplement.

We have reported earlier (Day *et al.*, 1959) that, under the experimental conditions employed in the paired feeding experiment (Series E), BHT produced a reduction in food consumption during the first one to two weeks after the weanling rats were placed on the diets containing BHT. However, by the fifth week no significant differences in food consumption were observed even between those animals whose diets contained 0·5 p.c. BHT, and no BHT respectively. The animals presumably had become "adapted" to the peppery nature of the diet containing BHT. Fig. 1 summarizes the effects of BHT on the weight of male rats six weeks after being placed on the BHT-containing diets used in the paired feeding experiment. Even though there were significant differences in the food consumption during the first portion of the experiment, at six weeks there were no significant differences in weight between the various control animals. Therefore the weights of all the control animals on the same lard level have been pooled. As this was a paired feeding experiment, all animals on the same lard level consumed the same amount of food; the results confirmed the rôle of increased lard content in the diet enhancing the deleterious effects due to BHT. A significant reduction in the weight at six weeks was again observed in male rats whose diet contained the NTD of BHT with a 20 p.c. lard supplement. Since this effect of BHT could not now be explained on the basis of reduced palatability of the diet due to the inclusion of BHT, it indicated a toxic effect.

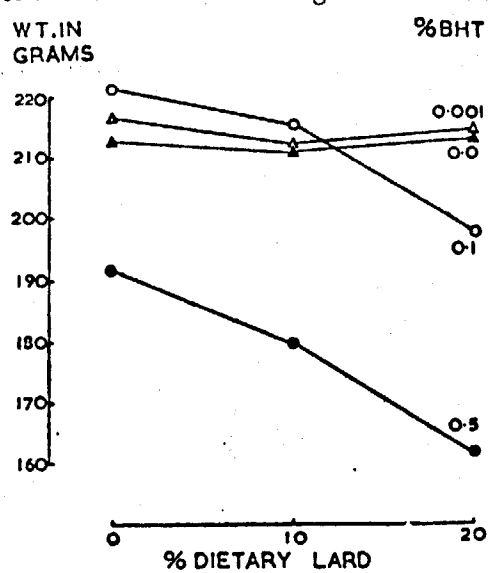


**Fig. 1.** The effect of a six-week dietary regimen of lard and BHT on the weight of pair-fed male rats.

The initial growth rate of male rats was increased by a 0·001 p.c. supplement of BHA in the diet containing 10 p.c. lard (Table 3, Series B), and in both male and female rats by 0·1 p.c. BHA in the diet containing 10 p.c. hydrogenated coconut oil (Table 3, Series A). This effect was abolished by heating the lard containing BHA. We have shown (Johnson *et al.*, 1958) that some 60 p.c. of the BHA in a lard solution containing 0·001 p.c. BHA is lost by heat destruction.



The NTD of BHA had no adverse effect on the initial growth rate of the rat. A diet containing 0·5 p.c. BHA produced only a relatively small reduction in the initial growth rate as compared with a diet containing 0·5 p.c. BHT. The reduction in the initial growth rate produced by 0·5 p.c. BHA was abolished by the heat treatment.

*Mature weight.*

The effect of BHT on the mature weight of the rat (Table 3) was very similar to that on the initial growth rate. Again, interactions were observed between Sex X BHT and Lard X BHT. The NTD of BHT produced a reduction in the mature weight of male rats when fed in conjunction with a 20 p.c. lard supplement.

In the case of the BHA fed animals there was no close correlation between the initial growth rate and the mature weight. Thus, although 0·001 p.c. BHA stimulated the initial rate of growth, no differences were observed in the mature weight. The normal testing dose of BHA did not produce any deviation in the mature weight. In some cases a small reduction in the mature weight occurred when the diet contained 0·5 p.c. BHA.

*The effect of BHT on hair.*

Shortly after being placed on the diets containing BHT a number of both male and female rats of series C and D showed a browning and thinning of the hair between the ears, which continued until it produced a definite bald patch approximately 20 mm. in diameter. This localized loss of hair extended down the flanks in some rats on the 0·5 p.c. BHT diet. In the majority of cases, the hair was regenerated within six weeks even though the animals were still on the diets containing BHT. A similar loss of hair occurred at a second stage in the life cycle of the female rats fed BHT, one or two weeks after cessation of weaning of young.

When the diet contained 0·1 p.c. BHT and 10 p.c. hydrogenated coconut oil (Series A), 8 out of 24 rats had bald patches, while there was no evidence of baldness in 22 control rats. The result was highly significant ($p < 0·01$).

In series D an attempt was made to assess quantitatively the rôle of the level of dietary lard in the development of baldness (Table 4).

The baldness index was assessed on an arbitrary scale which depended upon the duration and severity of the loss of hair. An increased level of lard in the diet enhanced the depilatory effect of BHT. No sex difference in the time of onset of baldness could be shown. In Table 4 the apparent sex differ-

TABLE 4.

*The effect of BHT and lard on the "baldness index" of the Norway Hooded rat.*

| Sex | p.c. BHT in diet with 10 p.c. lard supplement | | | p.c. BHT in diet with 20 p.c. lard supplement | | |
|---|---|---|---|---|---|---|
| | 0·0 | 0·1 | 0·5 | 0·0 | 0·1 | 0·5 |
| Male | 0 | 14 | 45 | 0 | 41 | 51 |
| Female | 3 | 58 | 74 | 18 | 94 | 151 |

ence is due to the inclusion of the figures from the second occurrence of baldness in the female rat after lactation.

The depilatory effect of BHT was not due to external contamination of the skin by the food. No baldness occurred when, at weekly intervals for three months, lard or lard containing 0·5 p.c. BHT was applied to the hair between the ears in two groups of fifteen Norway Hooded rats. The effect might be specific to the black hair of the Norway Hooded rat, since no baldness occurred in Albino rats fed on a diet containing 0·1 p.c. BHT in 10 p.c. hydrogenated coconut oil for six months. No such loss of hair has so far occurred after eighteen months in an experiment, at present in progress, on the effect of BHT on the reproductive capacity of the mouse.

Impressions and whole mounts (Benedict, 1959) were taken of hairs in the vicinity of the baldness from an experimental animal which had lost hair, an experimental animal that had lost hair but whose hair had regenerated, and from a control animal. These showed no differences in the distribution of the pigment granules, the medullae or of the structure of the scales. It can be concluded that the effect is not related to any change in the gross structure in the hair.

Histological examinations of sections of the skin of all animals in series D showed the typical variations in follicular activity which are characteristic of the hair cycle in most species of mammal (Flesch, 1954). The experimental conditions had no significant effect on this cycle.

BHA had no effect on the condition of the hair of either the Albino or Norway Hooded strain of rat.

*Organ weight studies.*

Smyth *et al.* (1952) have established that a comparison of organ weights relative to body weight is a sensitive index to stresses being placed upon experimental animals. Organ weight studies were carried out on experimental series B, D and E. BHA and BHT had no effect on the relative weights of the spleen, heart and kidneys. In the case of the testes it was shown that, in the normal adult rat, there was little correlation between the weights of the testes and the body. On diets containing BHA and BHT the animals had the same mean



absolute testes weight as the controls. As there was a reduction in the body weight of those animals whose diet contained the highest concentration of antioxidant, an increase in the ratio of testes to body weight was produced. This increase could be accounted for entirely by the decrease in the body weight. Both antioxidants did produce increases in the weight of the liver (Table 5). As no interactions were encountered, the values for the sexes, lard levels, and effect of heating the lard have been pooled.

TABLE 5.

*The effect of BHA and BHT on liver weight of rats.*

| Antioxidant | Experiment series | Measurement | Dietary level of antioxidant (p.c. dry weight) | | | |
|---|---|---|---|---|---|---|
| | | | 0 | 0·001 | 0·1 | 0·5 |
| BHA | B | RLW† | 3·28 | 3·17 | 3·38 | 3·69*** |
| | | ALW‡ | 7·89 | 7·76 | 8·10 | 8·30 |
| BHT | D | RLW | 3·73 | — | 4·00** | 5·17*** |
| | | ALW | 9·30 | — | 9·91 | 10·47 |
| | E | RLW | 3·51 | 3·40 | 4·04*** | 5·29*** |
| | | ALW | 7·50 | 7·28 | 8·73 | 9·54 |

† RLW = Relative liver weight in gm. liver per hundred gm. body weight.
‡ ALW = Absolute liver weight gm. (not analysed statistically).
*, **, *** Indicates logarithm of figure is significantly different from control with p = 0·05, 0·01, 0·001 respectively.

*Histological studies.*

Sections were prepared from the spleens, testes, livers, kidneys and aortae, in the D series; and similar sections were made from these tissues in a random selection of the animals of the B series. These sections were examined by a histologist who had no prior knowledge of the dietary history of the animals. No differences were observed between control and test specimens of the spleens, testes, kidneys or livers.

Lesions were observed on many of the aortae of series D. They were localized to the thoracic end of the aorta, particularly the aortic arch, no lesions were noted in any other segment. The lesions appeared macroscopically as small round pearly white nodules up to 2 mm. in diameter, and, histologically, were typically atherosclerotic, consisting of hyaline fibrocellular intimal proliferation, extending into the media, in which a complete disorientation of the medial architecture was apparent. Some of the lesions contained a small number of foam cells on the surface, but these were not a prominent feature. Several small areas of medionecrosis were seen. No calcification was noted in the atheromata which were also devoid of any inflammatory cell infiltration. Subintimal fragmentation and disruption of the medial muscle and elastic fibres were always found in

these lesions. A moderate hyaline fibrocellular intimal thickening was noted in many of the aortae. This change was maximal in the thoracic segment of the aorta. It was not modified by sex or dietary supplements.

In many of the atherosclerotic lesions there were focal and discrete accumulations of metachromatically-staining intercellular material. Such focal accumulations in and subjacent to lesions were clearly in excess of the metachromasia noted in the adjoining normal aortic segments. There was also a focal increase in PAS positive material in, and subjacent to, many of the atherosclerotic lesions. Both metachromatically-staining material and PAS positive material were more intense in the thoracic end of the aorta than in the abdominal end.

In the male rats receiving BHT, there was a considerable reduction in the amount of PAS positive material present in the aortic wall. There was no appreciable difference between groups receiving the two dietary levels of BHT with respect to the amount of PAS positive material present. In sharp contrast to the reduction in aortic PAS positive material in the male rats, was the absence of any such reduction in the female groups.

In general the incidence of aortic lesions was greater in the male than in the female animals. In the male rats there was a tendency towards reduced severity of atherosclerosis in those animals receiving BHT.

*Reproductive studies.*

The effect of BHA and BHT on one reproductive cycle of the rat is shown in Table 6.

Statistical analysis by the methods of Bruce and Emmens (1948) on the number of pups born and weaned, and on the total weight of the litter at 21 days, revealed no differences between treatments.

*Anophthalmia.*

In series D, three out of the thirty litters contained anophthalmic (uni-and bi-lateral) young when the parents had been fed BHT for five months. Although the numbers involved were small, it should be pointed out that anophthalmia has not been observed in any of our stock or other experimental rats. The number of anophthalmic young to the total number in the three litters was 1/11, 1/11, and 8/11. A dissection and histological examination of the heads of the anophthalmic animals showed a complete absence of eyeball but the presence of an undeveloped optic nerve. An attempt was made to differentiate between a genetic or dietary causal factor. Upon reaching maturity the litter mates of the second generation of these three litters were mated, using as many as possible combinations of diet with or without BHT, and normal or anophthalmic animals. The mating of anophthalmic male and female litter mates failed to produce anophthalmic young even though the diet in some cases



TABLE 6.

*The effect of BHA and BHT on one reproductive cycle of the rat.*

| Antioxidant | Dietary level and type of fat | p.c. Dietary level of antioxidant | No. of females | Average No. of pups | | Average Weight at 21 days —gm. |
|---|---|---|---|---|---|---|
| | | | | Born | 21 days | |
| BHA | 10 p.c. hydrogenated coconut oil | 0·0 | 12 | 6·8 | 2·4 | — |
| | | 0·0 | 13 | 6·8 | 3·4 | — |
| | 10 p.c. lard | 0·0 | 10 | 8·9 | 7·5 | 31·4 |
| | | 0·001 | 10 | 11·3 | 10·6 | 26·8 |
| | | 0·1 | 10 | 11·0 | 9·4 | 27·8 |
| | | 0·5 | 10 | 9·2 | 6·8 | 25·8 |
| | 10 p.c. heated lard | 0·0 | 10 | 9·9 | 7·6 | 28·6 |
| | | 0·0004 | 10 | 8·9 | 8·0 | 29·5 |
| | | 0·1 | 10 | 9·6 | 8·0 | 30·1 |
| | | 0·5 | 10 | 10·7 | 8·9 | 24·9 |
| BHT | 10 p.c. hydrogenated coconut oil | 0·0 | 11 | 8·8 | 6·5 | — |
| | | 0·1 | 12 | 8·3 | 7·6 | — |
| | 10 p.c. lard | 0·0 | 8 | 10·0 | 5·4 | 32·3 |
| | | 0·1 | 8 | 9·4 | 7·2 | 30·7 |
| | | 0·5 | 8 | 8·0 | 7·0 | 30·1 |
| | 20 p.c. lard | 0·0 | 7 | 10·1 | 8·5 | 31·8 |
| | | 0·1 | 8 | 10·2 | 8·1 | 34·1 |
| | | 0·5 | 8 | 9·8 | 8·7 | 28·8 |

contained 0·5 p.c. BHT. The only case where blind offspring were produced was when a blind male on 0·5 p.c. BHT was mated with a normal female on the same diet.

With the small numbers involved it was not possible to trace the cause of the anophthalmia. It is unlikely that a recessive gene was responsible, as the mating of anophthalmic sibs should have produced anophthalmic young. In the mouse reproduction study already mentioned some 600 litters have been produced from animals whose diet contained BHT, and in no case was anophthalmia observed.

*Mortality studies.*

Because of a considerable decline in the condition of both control and experimental animals, the survivors of experimental series A and C were killed after two years on the diet. A gross pathological examination revealed no differences between the control and experimental animals. An $\chi^2$ test of the number of deaths in the experimental groups as compared to the control groups showed that neither BHT nor BHA produced a significant increase in mortality after two years (Table 7).

TABLE 7.

*The effect of 0·1 p.c. BHA and 0·1 p.c. BHT in 10 p.c. hydrogenated coconut oil on survival to two years by the rat.*

| Antioxidant | | Died | Survived |
|---|---|---|---|
| BHA | Control females | 6 | 7 |
| | Control males | 7 | 6 |
| | Experimental females | 7 | 6 |
| | Experimental males | 10 | 3 |
| BHT | Control females | 4 | 8 |
| | Control males | 2 | 10 |
| | Experimental females | 5 | 7 |
| | Experimental males | 7 | 5 |

No autopsies were carried out on animals dying during the course of the experiment, as in the majority of cases death occurred at night and post-mortem changes were too advanced. In the experimental series B, D and E the percentage of deaths before completion of the experiment was very small, and in no case could be attributed to the treatment applied.

*Urine.*

In the experiments in which the food consumption was measured and the urine was absorbed on blotting paper placed in tin containers, the urine of animals on BHT turned a bright pink. However, when BHT fed animals were placed in metabolism cages and the urine collected in glass vessels, the urine was of a normal colour. The colour change probably resulted from the metal catalysed oxidation of BHT or its metabolites.

## DISCUSSION.

Before approval is granted to use an additive in human food, the Food and Drug laws of most countries usually require detailed evidence of non-toxicity to experimental animals when the additive is administered at an arbitrarily defined dietary level 100 times the maximum normally expected to be present in human food. The possibility that interactions with other components of the diet might affect the toxicity of the substance under consideration has sometimes been overlooked (Wilson and DeEds, 1950).

Under the conditions of this study BHA fulfilled the requirement of safety, as only normal cellular behaviour was apparent at the NTD. The stimulation of growth produced by BHA at the 0·001 p.c. level in male rats with unheated lard as the carrier fat, and at the 0·1 p.c. level using hydrogenated coconut oil, may possibly be related to the protection of easily autoxidizable constituents of the diet. It is unlikely the effect is due to protection of substances in the diet before its consumption as the diets were freshly prepared daily, and the dispensed lard, and lard containing antioxidant were prepared weekly and stored at 0° C.



However, BHT fed at the NTD in conjunction with a 20 p.c. lard supplement, but not with a 10 p.c. lard supplement, reduced the initial growth rate of male rats. No reduction was observed in female rats. Increased lard content of the diet enhanced the deleterious effect of this antioxidant on loss of hair.

The results of the paired feeding experiment showed that the reductions in the weight of the animals at the NTD with a 20 p.c. lard supplement were a direct toxic effect of BHT, and were not due to a restriction of food intake arising from the unpalatable nature of the diets containing BHT.

A further indication of the toxic effect of BHT at the NTD was the increase in the relative weight of the liver at this dietary level. Because of the very close correlation between liver and body weights, it was not considered possible to analyse the absolute weights of the liver; in the case of the BHT fed animals there appeared to be an increase in the mean absolute weight of the liver. BHA at the NTD had no effect on the relative liver weight, but an increase in this value occurred when the diet contained 0·5 p.c. BHA. There were no abnormal histopathological changes associated with the increase in relative weight of the liver. Dacre *et al.* (1956) and Dacre (1957) have shown that BHA and BHT are to some extent excreted as glucuronides and ethereal sulphates. It is probable that the detoxication of these phenols occurs in the liver, and that the hypoplasia of the liver represents an attempt on the part of this organ to meet the stresses placed upon it by the ingestion of these unphysiological substances.

Further evidence that BHT was toxic at the NTD was the occurrence of a significant ($p < 0·1$) balding effect in the Norway Hooded strain of rat. The effect was not observed in albino rats nor in mice and could not be associated with an external depilatory effect. The loss of hair was noted only in animals undergoing a period of stress, i.e. during the phase of rapid growth in young male and female animals, and in the female animals after the cessation of weaning young. As there was a regeneration of the hair when these stresses were removed, it is likely that the animals' tolerance to BHT varied at different stages of the life cycle. Loss of hair can be caused by a variety of nutritional and endocrinological factors; and from the available evidence it is not possible to locate the primary lesion responsible for it. The localization of the loss of hair to the top of the head, except with a few animals on the top concentration of BHT, suggests an endocrine effect although Cunha *et al.* (1943) have shown that the loss of hair caused by inositol deficiency started in this position and then proceeded down the flanks. A possibility that cannot be excluded at this stage is that the loss of hair might be a secondary effect due to a partial sulphur deficiency caused by the excretion of ethereal sulphates, one of the products of detoxication of BHT (Dacre, 1957). The ethereal sulphate production might interfere with cystine metabolism which is of importance in the development

of hair. However, hair loss due to a dietary deficiency usually involves changes in the structure of the skin or hair. This was not apparent in the loss of hair produced by BHT.

At the concentrations used, BHA and BHT had no effect on one reproductive cycle of the rat. Even though anophthalmia has not been observed in any of our stock or other experimental animals, from the evidence available it is not possible to relate unequivocally the anophthalmia, present in the young of parents which had been fed BHT, to the presence of BHT in the diet.

The only consistent histopathological abnormality observed in this study was the appearance of typical atherosclerotic lesions in the male and female rats fed lard or lard plus BHT. Jones *et al.* (1957) have found coronary atheroma in rats on a dietary supplement of lard. Humphreys (1957) has found atheroma to occur spontaneously in a variety of this species.

The fat content of various national diets varies enormously, but English-speaking peoples of European descent consume about 40 p.c. of their dietary calories as fat (Yudkin, 1957). This is a mean figure and there must be many people consuming considerably more than this. It is reasonable to assume the two levels of fat fed in the experimental work reported in this paper fall within this upper range. Consequently, the toxic effects found in the animals fed BHT at the NTD on both dietary fat levels form a prima facie case against the use of BHT in human diets. It is very doubtful whether the fat content in the diet in the experiments of Deichmann *et al.* (1955) exceeded 5 p.c. (w/w) and the lack of toxicity reported in that study when BHT was fed at the NTD reinforces the conclusion reached in the present experiments that the toxicity of BHT is a function of dietary fat level. On the other hand, no evidence was found that BHA is similarly affected, and the results of these experiments support the general view that BHA is suitable for use in human diets (Wilder and Kraybill, 1948; Graham *et al.*, 1954; Graham and Grice, 1955).

Other workers have reported that addition of fat to the diet can alter the toxicity of a substance. Fats added to the diet increased the toxicity of benzene administered as a vapour (Li and Freeman, 1945), of alloxan injected intravenously (Houssay and Martinez, 1947), and of dietary dichlorodiphenyltrichloroethane (DDT) (Sauberlich and Baumann, 1947). The latter authors suggested that the fat aided in the absorption of DDT. Fat augmented the acute toxicity and decreased the chronic toxicity of derris (Ambrose *et al.*, 1943) and decreased the toxicity of cyanide (Meyer, 1939). Whether the increased toxicity of BHT with increased dietary fat contents reflects increased absorption of BHT or a more intimate metabolic relationship between BHT and fat remains to be shown.

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# CORRESPONDENCE

## Food Antioxidants in Tissue Culture

SIR,—Miss Milner has described some very interesting experiments on the effects of butylated hydroxytoluene (BHT) and related compounds on monkey kidney cells in tissue culture[1], but it is a pity that she should have balked at the task of interpreting her findings in terms of the safety in use of BHT as a food antioxidant. Having spoken in three successive sentences of p.p.m. in individual items of food for human consumption, mg/kg body weight doses in animal experiments and p.p.m. in tissue culture media, without suggesting how these might be inter-related, she abdicates all responsibility by concluding that " . . . it is difficult to make a direct comparison with the *in vivo* experimental results because of the different effects measured".

Of course the selection of parameters to measure is at the discretion of the experimenter, but another source of difficulty in this instance is the absence of any data on the kidney concentrations of BHT in animals receiving BHT in the diet.

Because unchanged BHT is not excreted in the urine of rabbits receiving doses of up to 1 g/kg body wt[2] it seems unlikely that the kidney concentrates this compound. We are, however, in the process of investigating this aspect of BHT metabolism.

In the meantime, reported results from liver analyses may be of interest. Daniel and Gage[3] found that rats given 5,000 p.p.m. BHT in the diet averaged less than 2 p.p.m. BHT in the liver. Thus the amounts of BHT in the diet required to produce liver concentrations of 7·5–30 p.p.m. (those used by Miss Milner) would exceed the $LD_{50}$ of this compound in the rat[4]. Legal tolerances would allow an average BHT content of up to 4 p.p.m.[5] in the human diet. In fact, the present dietary concentration in Great Britain is unlikely to exceed 1 p.p.m. If the intake and liver content of BHT have the same relationship in man as they do in the rat, the human liver would currently contain about $3 \times 10^{-4}$ p.p.m.

We hope that these comments may help in the interpretation of the tissue culture data[1] and in the design of further experiments to help us bridge the gap between animal studies and the human situation. But progress in this direction will only be possible if the results obtained with tissue cultures are considered against the background of toxicological data already in our possession.

Finally, one detail calls for correction. While it is true as stated[1] that BHT is permitted for use in butter, this permission only applies to butter for manufacturing purposes[6,7]; this means that the addition of BHT to butter for retail sale in Great Britain is illegal.

Yours faithfully,

D. GILBERT
A. D. MARTIN

The British Industrial Biological Research Association, Carshalton, Surrey.

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# RESEARCH ON THE TOXICITY OF BUTYLHYDROXYTOLUENE (BHT)

RU-5475

by

Dr. A. Sporn and Dr. O. Schöbesch

Work done in the Food Hygiene Section of the Institute of Hygiene and Public Health, Romanian People's Republic

* * *

Butylhydroxytoluene (BHT) is an antioxidant of the phenolic group, used in several countries (U.S.A., England, France, Sweden), for the preservation of fats [5].

In our country, the Institute for Food Research has been investigating the antioxidant effect and the possibilities of practical application of BHT. On the advice of the Ministry of Health and Welfare it has sought the assistance of the Institute of Hygiene and Public Health to establish the toxicity of this antioxidant.

Published data indicate that the acute toxicity of BHT is small, and that no symptoms of poisoning were apparent in dogs and rats after continued use of the substance. Thus, BHT in a concentration of 0.17-0.97 g/kg body weight was given to dogs for 12 months, and 0.8% BHT was added to rats' food for 24 months without any evidence of pathological modifications being found [5].

During extensive research in the U.S.S.R., rats have received 4-8 mg/kg body weight and dogs 4 mg/kg body weight of BHT for 1 year; they have shown no changes in their overall condition, body weight, blood picture, proteinemia, urine composition, activity of the blood catalase, peroxidase and cholinesterase, and the anatomicopathologic examination was negative. Nor have any modifications been found in the reproductive function or in certain blood indicators for 3 generations of rats [2].

These results indicate that BHT has a low toxicity, and, as such, could be used as antioxidant in the preservation of fats. However, research conducted by the same group in the U.S.S.R. weakens this conclusion with respect to the subacute toxicity of BHT. In these studies, white rats received 250 mg/kg body weight of BHT for 68-82 days; they developed more poorly, showed a lessened activity of the blood catalase, peroxidase and cholinesterase, disturbances in the synthesis of the phospholipids, and an accumulation of fats in the liver [1].

With these data in mind, we deemed that additional research was necessary before accepting the use of BHT as an antioxidant for fats.

To this end, the acute toxicity of the substance was investigated in a previous work; the DL50 was established by its intraperitoneal injection in mice, oral administration to rats, and introduction into the culture medium of Daphnia. Additionally,

observations were made of the influence of the substance on the overall condition, body weight, weight of the adrenal glands, vitamin C content of the adrenal glands, activity of the blood catalase and peroxidase, and blood picture of white rats, as well as of the sensitizing action of the substance on guinea pigs.

The results that were obtained indicate that although BHT has a low toxicity, it is not without influence on the organism [3].

In this study we have considered the subacute toxicity of the substance, using small doses; that is, the usual dosage or a dose that would not exceed 10 times the usual dosage.

## EXPERIMENTAL PROCEDURE

The experiments were conducted on 80 white rats, in individual cages, fed ad libitum a synthetic ration composed of 8% casein, 12% pork fat, 66% starch, 2% agar, 2% mixed salts, and the following vitamins: 0.25 mg each of thiamine, riboflavin, pyridoxine, and niacin, 1.5 mg of calcium pantothenate, 10 mg of choline, 30 mg of inositol, and 30 mg of para-aminobenzoic acid.

In some experiments a lower protein content was used in the ration; it was then replaced with starch to maintain the same caloric level in the ration.

In the 9% casein ration, no choline was added, so as to obtain a deficiency of lipotropic factors.

The following indicators were used to confirm subacute toxicity.

- Growth of the white rat, with attention to the development of the animals, weekly weight gain, and protein efficiency.
- Restoration of the hepatic proteins following fasting.
- Nitrogen content of the liver after prolonged administration of the substance.
- Lipid content of the liver after prolonged administration of the substance.
- Weight of the adrenal glands.
- Vitamin C content of the adrenal glands.
- Activity of the blood catalase.

These indicators have been used in accordance with the procedures of our laboratory, as described in previous papers [3, 4].

## RESULTS

1. Influence of the substance on the growth of the white rat. In the first experiment, 16 animals received a 19.9% casein ration, while the test group received 0.2% BHT; the influence on growth was noted for 8 weeks (Table 1).

The results obtained indicate that BHT has a positive influence on the growth of the animals; the final weight as well as the weekly gain in weight was larger in the test group than in the control group. Additionally, better food ingestion and protein efficiency were noted for the test group than for the control group.

Table 1

Influence of BHT on the Growth of the White Rat

| Group | Indicators | Initial weight, g | Weeks I | II | III | IV | V | VI | VII | VIII | Average weekly values |
|---|---|---|---|---|---|---|---|---|---|---|---|
| I Control | Development, g | 57.0 | 75.5 | 94.0 | 110.4 | 125.6 | 136.8 | 142.6 | 147.4 | 147.8 | 11.4 |
| | Ingestion, g | - | 41.4 | 50.9 | 57.5 | 61.5 | 60.1 | 57.1 | 60.3 | 50.5 | 54.9 |
| | Protein efficiency | - | 2.23 | 1.82 | 1.13 | 1.24 | 0.93 | 0.51 | 0.40 | 0.05 | 1.03 |
| II 0.2% | Development, g | 57.0 | 82.6 | 106.0 | 119.4 | 136.8 | 147.5 | 156.1 | 160.8 | 163.0 | 13.3 |
| | Ingestion, g | - | 51.3 | 58.8 | 61.6 | 60.9 | 58.5 | 58.0 | 58.6 | 50.5 | 57.3 |
| | efficiency | - | 2.27 | 1.81 | 0.99 | 1.30 | 0.83 | 0.67 | 0.36 | 0.20 | 1.15 |

These results do not agree with those of Karplyuk [1], who notes a decrease in the weight of rats under the influence of BHT. These differences could also be due to the different dosages used in the two experiments. Thus, while we stimulate growth, using small doses of the substance, Karplyuk, using large doses, inhibits it. We have observed such phase behavior of chemical substances in other, previous research [4].

Although our hypothesis could explain the dissimilarity of our results, we considered it necessary to verify the experiment, in order to see the extent to which the observed phenomenon could be reproduced.

The second experiment was also performed on 16 white rats; their weight was comparable to that of the animals in the first experiment (59.0 g); they were similarly divided into two groups receiving a ration of 16.6% casein, with the same concentration of BHT in the test group. The results obtained at 4 weeks not only confirm the conclusions of the first experiment, but show even more marked differences. Thus, the final weight of the test group was 47% greater than that of the control group. The test group also showed a correspondingly good food ingestion and protein efficiency.

Considering that in our previous research we were able to sensitize the growth yardstick by decreasing the protein content of the ration, we also investigated the influence of BHT on growth for a ration of 9.6% casein. The experiments were performed on 16 white rats of comparable weight (56.5 for the control group and 56.6 for the test group). The results obtained show that at 7 weeks, the average values of the weight of the test group animals exceeds by 41% those of the control group. Comparable differences are also noted for the food ingestion and protein efficiency.

Thus, in 3 experiments, performed under various conditions, a better growth was recorded for the animals under the influence of BHT; this fully confirms the reproducibility and validity of the phenomenon.

2. Restoration of the hepatic proteins after fasting. The experiments were performed on 16 adult white rats, divided into two groups. During the restoration period the animals received a synthetic ration containing 19.9% casein for the control group and 21.7% for the test group. The ration of the test group contained 0.2% BHT.

The results obtained (Table 2) show that the test group had a nitrogen content in the liver smaller than that of the control group, even though it also had a better food ingestion during the realimentation period and a somewhat greater final weight than the same control group. Thus, the average values for the absolute quantities of nitrogen in the liver are 263 mg for the control group and only 229 mg for the test group; while the corresponding nitrogen content with respect to body weight is 145 mg% as compared to 127.4 mg%.

Table 2

Influence of BHT on the Restoration of Hepatic Proteins after Fasting

| | Weight, g | | | Nitrogen, mg | | Nitrogen, mg% |
|---|---|---|---|---|---|---|
| Groups | Initial | After fasting | After restoration | Ingestion | Liver | Body weight |
| Group I Control | 180.5 | 159.4 | 176.3 | 44.1 | 263 | 145.4 |
| Group II 0.2% BHT | 180.4 | 163.8 | 179.9 | 47.1 | 229 | 127.4 |

The results obtained show that BHT has a negative influence on protein synthesis in the liver.

3. Nitrogen content of the liver after prolonged administration of BHT. The determination of hepatic nitrogen was made on animals from the growth experiments, that is, on 64 white rats.

The results obtained (Table 3) show the groups receiving 0.2% BHT to have a lower nitrogen content in the liver, even though their weight at the time of sacrifice was greater than that of the control groups.

Thus, for instance, the average weight of the group II animals was 18 g higher than that of the control group, and the nitrogen content of their livers was 49.4 mg lower than that of the same control group; while with respect to their body weight, the nitrogen content was 115.4 mg% as compared to the 161.1 mg% of the control group.

Similar differences have also been seen in the other groups, as well as in the experiment of the restoration of hepatic proteins after fasting. This fact indicates the complete reproducibility and validity of the phenomenon; that is, of the negative influence of BHT on protein synthesis in the liver.

In the group that received only 0.02% BHT (group VIII), the nitrogen content of the liver was even a little higher than that of the control group. This fact indicates that BHT has a narrow margin of safety, since the dosage capable of causing important modifications in protein synthesis is only ten times larger than the one which produces none.

4. Lipid content of the liver after prolonged administration of BHT. The lipid content of the liver was measured on 48 white rats obtained from the growth experiments. The test group drawn from those groups receiving a 16.6% casein ration showed a tendency toward increase in the lipid content of the liver. Thus, while the average values of hepatic lipids in the control group are 0.348 g, viz., 7.46 mg per 100 g of body weight, the corresponding values are 0.433, viz., 8.1%.

Table 3

Influence of Prolonged Administration of BHT on the Nitrogen Content of the Liver

| Groups | Weight, in g | | BHT ingestion, mg | Mg of nitrogen in the liver | Nitrogen, mg% body weight |
|---|---|---|---|---|---|
| | Initial | At sacrifice | | | |
| I Control Casein 19.9% | 57.0 | 152.4 | - | 245.8 | 161.1 |
| II Casein 19.9% and BHT 0.2% | 57.0 | 170.4 | 916 | 196.4 | 115.4 |
| III Control Casein 16.6% | 59.1 | 88.5 | - | 159.7 | 184.0 |
| IV Casein 16.6% and BHT 0.2% | 59.0 | 120.7 | 452 | 150.9 | 125.3 |
| V Control Casein 9.6% | 56.5 | 96.3 | - | 111.1 | 115.9 |
| VI Casein 9.6% and BHT 0.2% | 56.6 | 136.4 | 827 | 127.2 | 92.6 |
| VII Control Casein 19.5% | 56.0 | 117.8 | - | 185.0 | 157.1 |
| VIII Casein 19% BHT 0.02% | 56.0 | 129.1 | 53 | 210.0 | 162.4 |

Larger differences exist between the test and control groups that were given rations with a lower content of choline and proteins (9.6%). Thus, while the lipid

content of the liver in the control group is 0.1928 g, viz., 17.4 mg% of the body weight, the corresponding values for the group receiving 0.2% BHT are 0.3438, viz., 22.4%.

In the experiments where the test group received 0.02% BHT, there are smaller differences in the absolute lipid content of the liver, and no significant differences are found in the percentage values with respect to body weight. Thus, the average lipid content of the liver is 0.3156 g for the control group, and 0.3438 for the test group, viz., 17.1% and 16.6%.

The results above show that a 0.2% concentration of BHT causes an increase in the lipid content of the liver; this increase is even more evident for a ration with a low protein level and devoid of choline.

Our results indicate that a smaller increase in the quantity of lipids in the liver takes place under the influence of BHT than that described by Karplyuk [1]; this could be due to the different dosage used in the experiments, our doses being smaller than those used by Karplyuk.

5. The weight of the adrenal glands was measured on the groups that received 19.9% casein, and 0.2% BHT, respectively. The results obtained show the adrenal glands to be heavier in the groups that received BHT. Thus, the weight of the adrenal glands was 0.3113 g for the control group, and 0.3876 g for the test group.

6. The vitamin C content of the adrenal glands was measured on the same groups. The results obtained show that BHT produces a clear and significant increase in the vitamin C content of the adrenals. Thus, while the control group contained an average of 158.18 ± 5.96 γ of vitamin C in the adrenals, the test group contained 219.2 ± 7.20 γ. The differences between the groups are statistically significant ($t = 2.54$). The vitamin C content expressed in per cent of the body weight was 98.5 ± 9.66 γ% and 150.1 ± 6.54 γ%. In this case the difference between groups is even more significant ($t = 3.92$).

The increase in the vitamin C content of the adrenals under the influence of BHT indicates that the substance tested places a stress on the organism.

No significant differences are seen in the values of the blood catalase, as determined on the same groups. Thus, the average values of the catalase index were 3.16 for the control group and 3.07 for the test group.

The correlated results of our researches show that BHT has a toxic action on the organism; this action takes place when BHT is administered in a dosage exceeding 10 times the usual dose, that is, in a concentration of 0.2%. The toxic action of BHT becomes evident if we consider its negative influence on the protein synthesis of the liver, on the lipid content of the liver, as well as on the vitamin C content of the adrenal glands.

The stimulation of growth on the part of BHT must also be interpreted as a negative effect; on the one hand, because it is not paralleled by a corresponding increase in the nitrogen content of the liver, and, on the other hand, because it seems to be characteristic only of small doses of the substance. In larger doses, BHT causes a decrease of growth, as has been found by other authors [1].

These data indicate that the use of BHT as an antioxidant for fats should be forbidden.

The need for such a ban on the use of BHT in food applications is also due to the fact that BHT can be placed in the category of substances with a narrow safety

margin. Its action on Daphnia, indicated by us in our previous investigations [3], is an intimation of this as is the fact that BHT has a distinct toxic effect for a dosage only 10 times larger than the nontoxic dose.

## CONCLUSIONS

1. The subacute toxicity of BHT was investigated on 80 white rats. Observations were made of: the development of the animals (weekly weight gain), food ingestion, protein efficiency, restoration of hepatic proteins after fasting, nitrogen and lipid content of the liver following prolonged administration (up to 8 weeks) of the substance, weight of the adrenal glands, their vitamin C content, and the activity of the blood catalase.

2. The results obtained indicate that a 0.2% dose of BHT, 10 times the usual dosage, produces a stimulation of growth, a poorer restoration of the hepatic proteins after fasting, a decrease in the nitrogen content of the liver following prolonged administration of the substance, an accumulation of fats in the liver, and an increase in the vitamin C content of the adrenals.

3. Considering BHT's toxic effect, as well as the low margin of safety it presents, we consider that it must not be used as a food antioxidant.

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# MONOGRAPH
# ON
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## SUMMARY

When BHT is ingested by man, it is readily absorbed and rapidly metabolized. Preliminary oxidation is generally followed by conjugation with glucuronic acid or glycine (6, 14). Elimination of BHT and its metabolites is a biphasic process (5): the first phase is rapid, with 50% of the dose appearing in the urine during the first 24 hours after administration (5); the slower, second phase probably represents the release of BHT or its metabolites from tissue (mainly adipose) storage (5). A biological half-life of 5 days has been determined for the portion of the BHT which is excreted during the second phase (5). Prolonged exposure to BHT does not cause progressive accumulation in the body tissues (4) nor alterations in its handling in man (17).

In rats, BHT consumption has been obsserved to affect the activity levels of several enzymes. The threshold for increases in liver processing enzyme activity has been found to be between 25 and 75 mg of BHT/kg/day for seven days (12, 13). An intake of 500 mg of BHT/kg/day for seven days causes depression of glucose-6-phosphatase activity and elevation of glucose-6-phosphate dehydrogenase activity (8, 11). No effect was observed on the activity of either enzyme when BHT was given at a dosage of 50 mg/kg/day for 16 weeks (10).

BHT causes an increase in the relative liver weight of test subjects (rats) when given for 16 weeks at a dosage of 50 mg/kg/day in the diet (10). At a dosage of 100 mg/kg/day, there is a slight proliferation of smooth endoplasmic reticulum in liver cells (9). This level of 100 mg/kg/day may be considered the limit of physiologic response, that is, as opposed to toxicologic response.

At dosages of 300 mg/kg/day and greater for 42 weeks, BHT has shown toxic effects: decreased growth rate and exaggerated relative liver and brain weights (9).

Despite administration of 500 mg/kg/day for 7 weeks, no teratogenic effects have been shown for BHT (15). Repeated experiments fail to show any effects on reproductive indices (18), lifespan (16), or effects on offspring (2) of a variety of test animals.

BHT has shown no carcinogenic activity (1). Indeed, recent studies show that it markedly inhibits the carcinogenic action of several polycyclic hydrocarbons (19, 20).

## SUMMARY TABLE

| Species | Length of Exp. | No Significant Toxic Effects | Min. significant Toxic Effects | Ref. |
|---|---|---|---|---|
| Rat | 16 weeks | 100 mg/kg/day | | 10 |
| Rat | 2 years | 400 mg/kg/day | 500 mg/kg/day | 7 |
| Rat | Multigeneration | 100 mg/kg/day | 300 mg/kg/day | 9 |
| Chicken | 1 year | 156 mg/kg/day | | 18 |

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BUTYLATED HYDROXYTOLUENE

Chemical Information

I. Nomenclature

A. Common names
butylated hydroxytolune
BHT
DBPC

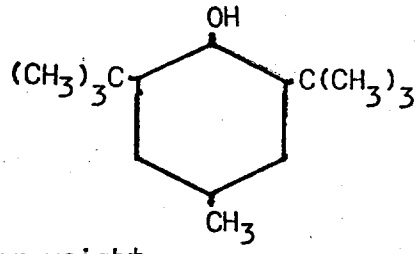
B. Chemical names
2,6-di-tert-butyl-p-cresol
4-methyl-2,6-di-tert-butylphenol
2,6-di-tert-butyl-4-methylphenol
3,5-di-tert-butyl-4-hydroxytoluene

C. Trade names
Ionol
Dalpac
Improvol
Vianol

D. CAS Registry Number - 000128370

II. Empirical formula
$C_{15}H_{24}O_{11}$

III. Structural formula




IV. Molecular weight
220.36

V. Specifications
A. Chemical
See C.
B. Food Grade
See C.
C. Food Codex
Not less than 99.0% C15H24011
Limits of impurities

| | | |
|---|---|---|
| Arsenic | Not more than | 3ppm (0.0003%) |
| Heavy Metals | Not more than | 10ppm (0.001%) |
| Ash | Not more than | 0.002% |

## VI. Description

### A. General

white, crystalline, odorless solid

### B. Physical properties

melting point - 70 deg.
boiling point - 265 deg.
Insoluble in water
Soluble in food oils fats methanol, ethanol, isopropanol, ethyl methyl ketone, acetone, cellosolve, petroleum ether, benzene and most hydrocarbon solvents

### C. Stability

0.01% BHT in animal fat reached 20 MEQ Peroxide in 20 hours (Control: 4 hrs.)
0.02% BHT in cotton seed oil reached 70 MEQ Peroxide in 12 hours (control: 8 hrs.)
0.02% BHT in corn oil reached 70 MEQ Peroxide in 15 hours (control: 12 hrs.) (51).

## VIII. Analytical Methods

A general method for the analysis of BHT and other antioxidants involves the solution of a sample in a suitable hydrocarbon solvent. This solution is then steam distilled and the BHT and BHA are separated from other antioxidants present and analyzed quantitatively. The sample solutions can also be separated by solvent extraction (5).

Quantitative methods depend on a colorimetric determination using a spectrophotometer. In the Emmerie-Engels Method the basis for the color formation is a reduction of ferric chloride reagent to the ferrous state by the antixoidant and the formation of an iron-bipyridyl complex (3). The Szalkowski-Garber Method, the first colorimetric method of analysis specific for BHT, depends upon the orange-red chromogen formed by a reaction involving dianisidine, sodium nitrate and BHT (6).

A systematic method to determine BHT and BHA in the presence of ethoxyquin is based on column chromatography. A sample of the food or feed is dissolved in a suitable hydrocarbon, such as heptane. To determine BHT the solution is passed through a Florisil chromatographic column. All antioxidants and other organic compounds except BHT will remain adsorbed on the column. The BHT in the solution is then determined by ultraviolet spectrophotometry (4).

A gas liquid chromatographic technique has been developed which requires about 20 min for the determination of BHA and BHT in vegetable oils (2). Soybean, cottonseed, corn and peanut oils fortified with 20, 60 and 100 ppm each of BHT and BHA showed a recovery range of 97 to 104%.

A thin-layer chromatographic method has been developed which can be used to separate and quantitatively estimate the four principle antioxidants most commonly used in foods These antioxidants are BHT, BHA, propyl gallate and nordihydroquaiarectic acid. The antioxidants were extracted with 80% ethanol and actonitrile from lard dissolved in n-hexane. After the solvent was evaporated and the residue redissolved in ethanol, the solution was spotted on thin-layer chromatography plates coated wiht Silica Gel-G. The plates were developed by two-dimensional chromatograph with benzene and acetonitrile and then sprayed with 2,6-dichloro-quinone chloride. The anitoxidant spots were removed from the plates and prepared for colorimetric determination using appropriate reagents. They were analyzed spectro-photometricly and showed a recovery range of 98 to 101% (1).

VIII. Occurrence

BHT is a synthetic compound prepared from p-cresol and isobutylene.

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## Biological Data

### I. Acute Toxity

TABLE I

| SUBSTANCE | ANIMAL | NO. & SEX | ROUTE | DOSAGE | MEASUREMENT | REFERENCE |
|---|---|---|---|---|---|---|
| BHT | Mouse | 5M, 5F | IG | 2000 mg/kg | $LD_{50}$ | 9 |
| BHT | Mouse | 10M | IG | 836-1244 mg/kg | $LD_{50}$ | 11 |
| BHT | Mouse | 5M, 5F | IG | 2500 mg/kg | $LD_{100}$ | 9 |
| BHT | Rat | 4M, 4F | IG | 500 mg/kg | MTD | 9 |
| BHT | Rat | 4M, 4F | IG | 2450 mg/kg | $LD_{50}$ | 9 |
| BHT | Rat | 42M | IG | 1700 mg/kg | $LD_{50}$ | 4 |
| BHT | Rat | 42F | IG | 1970 mg/kg | $LD_{50}$ | 4 |
| BHT | Rat | 4M, 4F | IG | 3500 mg/kg | $LD_{100}$ | 9 |
| BHT | Guinea pig | 3M, 4F | IG | 10,700 mg/kg | $LD_{100}$ | 4 |
| BHT | Rabbit | 6M, 6F | IG | 2100-3200 mg/kg | $LD_{100}$ | 4 |
| BHT | Cat | 9M, 9F | IG | 940-2100 mg/kg | $LD_{100}$ | 4 |

### II. Short Term Studies

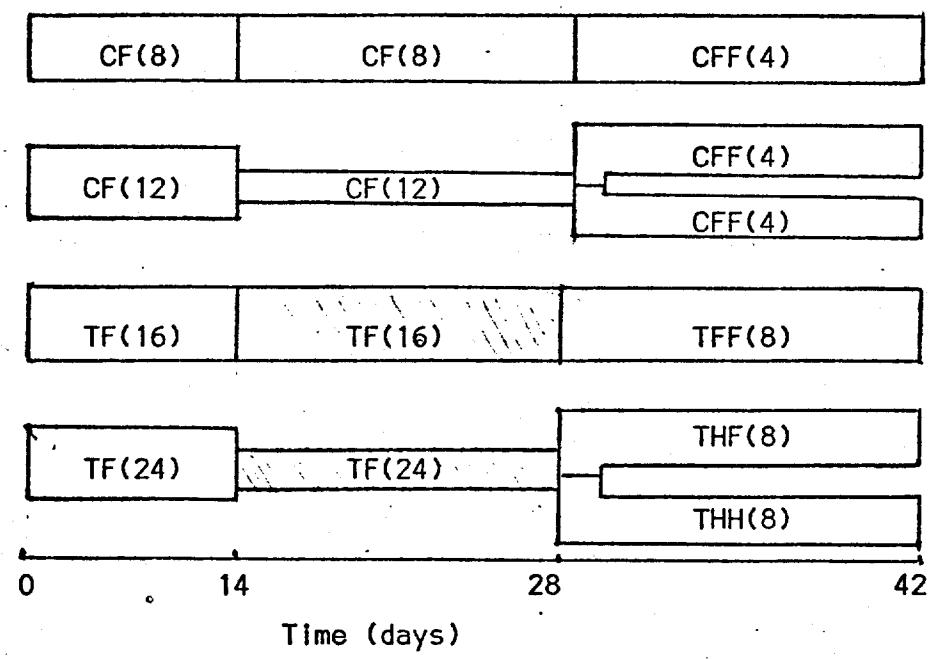
Rats

Two groups of 48 weanling Carworth SPF rats (24 females, 24 males) were used in a feeding study in which one group was maintained as a control and the other received 0.1% BHT/day in a ground diet. Six rats from each group were sacrificed at 4, 6, 12, and 16 weeks, with daily urine samples being collected in TCA for 4 days before. At autopsy, major organs were weighed and liver samples were taken for biochemical determinations (6) (see biochemical section).

The growth of BHT-fed animals at this level (0.1% BHT/day) closely parallel that of the control subjects. The only significant changes noted were increase of the organ weight/ body weight for liver (females at 4, 6, 12, 16 weeks; males at 4, 12 weeks) and adrenals (females at 12 weeks). Gaunt noted that the presence of BHT in the diet, particularly at high levels (250-500 mg/kg/day), rendered it impalatable to rats and thereby introduced a parameter of stress in growth determination (6).

SPF weanling rats, housed in pairs, were divided into 2 control and 2 test groups as shown:

TABLE II




First letter C, T indicate control and BHT-treated respectively. Second letters, F, H denote full and restricted diet during treatment phase. Third letter F, H denote same during necessary phase. Shading indicates treatment (500 mg BHT/kg/day) administered by intubulation as 10% (w/v) solution in arachis oil.

After a 7 day acclimatization period, data were obtained on urinary ascorbic acid excretion and growth rate from day 7 to day 42. Several biochemical regimens were executed (see biochemical section) (7).

Despite massive dosages of BHT, there was no significant growth retardation. BHT did not affect the relative weight of the adrenals but did increase relative liver weight. This effect was not apparent, however, after the 2 week recovery period (7).

On the basis of studies with hooded rats (Rattus norvegicus), Brown, et.al., found that BHT at a dosage of 0.1% BHT/day in a 20% lard-base diet produced a significantly reduced growth rate as well as depiliation of the head (2).
Subsequent work has failed to confirm this, and consensus among workers is that the effect may have been the result of A-vitaminosis (3).

Chickens

Twenty white leghorn pullets (18 weeks old) were used with 10 held as control animals and 10 fed 0.125% BHT/day in their diet. Three months after pullets came into production, eggs were collected and yolks analyzed for Vitamin A. After 4 months of production, the pullets were artificially inseminated and eggs were collected to study the affect of BHT on hatchability. Production records and feed conversion records were kept; liver and blood serum samples were obtained at the end of the experiment (1 year) (12).

At 0.125% BHT/day for 1 year, BHT did not have an adverse affect on fertility, hatchability, viability of offspring nor did it affect the hens in any other apparent way.

Dogs

Four mongrel dogs were fed dosages of 11,200; 8,400; 19,200; and 37,600 mg BHT/kg/day respectively for a 4 week period in a ground meat diet. During the first week, the dogs responded with a mild to moderate diarrhea; during the second and third week, the onset of diarrhea was delayed; and, during the fourth week, only a mild diarrhea developed despite the heavy dosage. No other signs of intoxication were observed; upon autopsy, there was no sign of gastroenteric irritation (4).

A second group of 6 dogs were fed the following dosages of BHT for 260 days over a period of 1 year: 170, 230, 500, 470, 640, and 940 mg/kg. The BHT was mixed in lard and fed with ground meat. At the end of one year, the subjects were sacrificed and histopathological studies of major organs were performed. At the levels of test ingestion, no gross nor microscopic pathology is attributable to BHT (4).

III. Long Term Studies

Rats

For 2 years, 6 groups of 30 Wistar rats (15 females, 15 males)

were placed on diets containing 0.2, 0.5, 0.8, and 1.0% BHT/day in 1% lard respectively; one control group fed 1% lard; and, one special group fed 0.5% BHT/day in 1% lard dissolved and heated for 30 minutes at 150oC. The animals were weighed once a week until a constant weight was attained and monthly thereafter. Blood counts and hemoglobin determinations were executed at intervals (2). At the end of the test period, the rats were sacrificed (CHCl3) and examined for gross and micropathologic changes, and organ weights were determined (4).

There was no correllation between mortality and BHT. On the basis of 277 examinations, Deichmann reported no affect on erythrocytes, leucocytes, or hemoglobin levels. There was no effect on weight gain at 0.8%/day however, at 1%/day both females and males rats manifested a subnormal weight increase. At 0.8%/day there was no effect on organ weight; however, at 1.0%/day both brain and liver of both sexes increased in weight. No consistent pattern of anatomic pathology was observed over the long term period, even in those animals at the 1.0% BHT/day dosage level (4).

## IV. Special Studies

### Longevity

Two experiments on the effect of BHT on life-span of C57BL mice were carried out. In the first experiment, BHT was administered at 0.7%/day in the diet. Excessive mortality resulted; the dosage was lowered to 0.5%/day and again to 0.2%/day. No conclusion was reached (10).

In the second experiment, BHT was administered at 0.5%/day for 150 weeks. Control mortality was significantly greater then that of the BHT-fed animals. BHT lengthened lifespan under sub-optimal conditions and had no significant affect under optimal conditions (10).

### Multigeneration

Three experimental and 2 control groups (16 females, 16 males) of 21 day-old Sprague-Dawley albino rats were fed 20% lard diets containing 0, 300, 1000, and 3000 ppm BHT/day for their entire reproductive life. At 100 days of age, 16 females were mated with 16 males on the same dietary regimen. Fertility, parturition, and lactation were examined in the parents (females) and viability, survival, growth, and behavior in the offspring. At 28 weeks (10 days after weaning the first litter),the females were mated again and the same regimen executed on parents and offspring. At 42 weeks (after weaning of the second litter), these offspring were fed the same diet as their parents until 100 days of age and were mated in the same fashion. The same regimen was again executed on these animals and 2 litters of their offspring (5).

At the 3000 ppm level a 10-20% reduction in growth rate of parents and offspring was observed. Although no effect was noted after 10 weeks, an elevation of serum cholesterol was evident at 28 weeks and a 10-20% weight increase of the liver after 42 weeks. No effects of any kind were noted at the 300 ppm and 1000 ppm levels (5).

All reproductive criteria were normal at all levels of dosage. No teratogenic effects were observed (even at 3000 ppm BHT/day). This multigeneration study substantiated that 1000 ppm BHT/day on a high-fat (20%) diet is the no-effect level (5).

Carcinogenesis

Boutwell and Bosch using a common pool of mice (C3H, CAF, Sutter) 2-3 months old performed an experiment determining topical carcinagenicity of various phenol derivatives. One week prior to topical application of test solution, fur was shaved from the test area of the back with electric clippers. As 3% solution of 9,10-dimethyl-1,2-benzanthracene in benzene was applied (1 drop) mid-dorsally as an initiator, and a 20% solution of BHT in benzene was applied (1 drop) twice weekly for 15 weeks. Of 33 test subjects, 28 survived. No papillomas or carcinomas were observed (1).

Inhibition of Carcinogenesis

One group of 18 female Spraque Dawley rats was given 200 mg BHT in olive oil one hour prior to dosing orally with 12 mg of dimethyl-benzathracene. A control group of 34 rats was given olive oil and then dosed with DMBA. Eighteen weeks later, 79% of the control group had developed breast tumors; only 28% of the BHT-treated animals had (15).

Further studies using rats and mice as test animals showed that not only did BHT inhibit carcinogenic activity but also inhibited toxic effects attributable to DMBA (15).

In another study, Fischer and Charles River rats were fed 223 ppm N-2-fluorenylacetamide or 239 ppm N-hydroxy-N-2-fluorenylacetamide and 6600 ppm BHT in the diet. BHT reduced the incidence of hepatoma and mammary adenocarcinoma drastically (14).

Teratogenic

Litters of weanling mice (albino and anophthalmic strains) which contained at least 6 females and 1 male were selected and used to comprise 6 test groups, each group containing female and male sibs and 5 unrelated females. Three of the groups were used to test the effect of BHT at 0, 50, 250 mg/kg/day dosages for 18 months in 10% lard; the other 3 groups were used to test the effect of BHT at 0, 0.1, 0.5% BHT/day for 18 months in 20% lard

The BHT-laden lard was incorporated in the diet. Prior to parturition, females were trasferred to separate cages. Pups were observed at day 1, 5, and 12, and the female was returned to the group after 1 week in the nesting cage. This regimen was repeated throughout the reproductive lifespan. The experiment was terminated after 18 months (8).

Of 7765 mice born, none were anophthalmic. At 0.1%/day for 18 months, BHT had no statistically significant effect on gestation time, pup weight, litter weight, or litter mortality of the test mice. BHT is not a teratogenic agent (8).

Clegg performed several experiments on rats and mice of various strains. See table III:

TABLE III
SUMMARY OF ANIMAL GROUPINGS AND DOSAGE

| Exp.* | Strain | No. of Animals | Test Substance | DOSE IU | DOSE mg/kg | Dose regime |
|---|---|---|---|---|---|---|
| | | | MICE | | | |
| 1 | Evans No. 1 | 10 | BHT | | 750 | Daily administration for 32-46 days prior to mating and up to day 18 of pregnancy. |
| 2 | Evans No. 1 | 83 | BHT | | 1000 | Single administration on a specific day of pregnancy (day 1-18). |
| 3 | Evans No. 1 | 10 | BHT | | 750 | Daily administration on days 1-18 of pregnancy. |
| 4 | ICI SPF | 95 | BHA | | 500 | Daily administration for 7 wk before pairing, continuing until day 18 of pregnancy. |
| | | | BHT | | 250, 300, 500 | |
| | | | Vitamin A | 30,000 | | |
| | | | RATS | | | |
| 5 | Tuck albino | 32 | BHA | | 750 | Daily administration on days 1-20 of pregnancy.† |
| | | | BHT | | 750 | |
| | | | Vitamin A | 5000 | | |
| 6 | Tuck albino Benger hooded | 47 | BHA | | 750 | Daily administration for 70 days before pairing, continuing through pregnancy. |
| | | | BHT | | | |
| | | | Vitamin A | 5000 | | |
| 7 | Tuck albino Carworth SPF albino | 111 | BHT | | 1000 | Single administration on day 9, 11 or 13 of pregnancy. |
| | | | BHT | | 1000 | |
| | | | Vitamin A | 60,000 | | |
| 8 | Benger hooded Portion SPF albino | 152 | BHA | | 500 | Daily administration for 7 wk before pairing continuing during pregnancy; treatment commenced when rats were 3 or 10 wk old. |
| | | | BHT | | | |
| | | | Vitamin A | 30,000 | | |

*Including arachis oil control and untreated control.
†12 of these rats were allowed to continue to parturition, the offspring being examined at weaning (3 wk old).

BHT was administered in arachis oil by intubulation. Except in experiment 6, pregnancies were terminated prior to parturition (day 18 mice, day 20 rats). At termination of pregnancy of the rats, both ovaries and uterus were removed and corporea lutea of each ovary was noted. Number and position of implanation sites (including resorption sites) were recorded and foetuses removed, examined for gross abnormalities, sexed, weighed, fixed, and stained by two procedures for soft tissue examination and for skeletal abnormality examination (3).

This study failed to confirm the findings of Brown, et. al (2). Depiliation was not seen and foetal abnormalities were limited to a single case of delayed skeletal development (3).

Dermal

Using rabbits, McOmie, et.al., applied BHT topically on gauze to the skin for 24 hours. In another test, BHT dissolved in diethyl ether was flowed on to the shaved back of a rabbit. At the test dosage of 3300 mg/kg for 1 day, the BHT showed no evidence of irritant action. At a dosage of 420 mg/kg in ether solution some erythema and superficial sloughing resulted (11).

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## Biochemical Aspects

I. Breakdown

No information available

II. Absorption-Distribution

When given orally, BHT is readily and completely absorbed into the intestine. Dissemination of BHT throughout the organism is found when the antioxidant is a regular part of the diet. Being lipid-soluble, the BHT which is deposited is found mainly in the adipose tissue. However, the level of BHT is independent of the duration of dosing and drops back essentially to zero when dosing is terminated indicating that the BHT deposition is of a dynamic nature.

In extended experiments on rats consuming daily up to 10,000 times the estimated human intake, the BHT level of the rat fat was found to initially rise as high as 200 ppm (days 2-3) and to level off (by day 7) at 100 ppm or less (34).

In the rate of absorption and the extent of BHT deposition, at low and intermediate levels of dosage, have been found to be dependent upon sex. The female rat absorbs BHT more readily than the male, resulting in a slightly higher level of BHT deposition in the fat of the female (8, 38).

In chickens given 200 ppm BHT in their feed for 1 to 10 weeks, BHT and its metabolites have been found in the edible portions at levels of 1-3 ppm. The eggs of laying hens fed BHT for 4 weeks contained 2 ppm of BHT and its metabolites (4). In another experiment, dietary levels of 5 and 50 ppm fed to laying hens for 8 weeks caused no accumulation of unchanged BHT in egg fat. When these dietary levels were increased to 100 and 500 ppm BHT, respectively, and the experiment continued for another 21 wks only a trace of unchanged BHT was found in egg fat at the 100 ppm and 24 ppm BHT at the 500 ppm dietary level (39).

When chickens were fed 0.1% BHT in their diet for eight weeks, an accumulation of 60 ppm BHT was found in the chickens' fat (13).

Samples of fat extracted from human subcutaneous adipose tissue were analyzed to determine the level of BHT accumulation. In six male and five female residents of England who had consumed an estimated 1 mg of BHT daily, the level of the lipid portion of the adipose tissue was between 0.01 and 0.49 ppm, with a mean content of 0.23 ppm and a standard deviation of 0.15 ppm. In seven male and five female residents of the United States who had consumed an estimated 2 mg of BHT daily, the BHT content of this lipid was between 0.34 and 3.19 ppm, with a mean content of 1.30 ppm and a standard deviation of 0.82 ppm. No sex-related differences in the BHT levels were observed in either country (4).

III. Metabolism and excretion

The metabolism of BHT is complicated by the presence of the t-butyl groups on each side of the hydroxyl. Either oxidation of one of the t-butyl groups to a 2-hydroxy-2,2-dimethyl-2-ethyl group, or oxidation of the methyl group to a carboxy acid must take place before conjugation can occur. The extent of these reactions and the resultant metabolites are dependent upon the species involved. The metabolites of the various species are listed in Table IV. The liver appears to be the major site of BHT metabolism.ab

Table IV
The metabolites of BHT. (primary metabolite listed first)

| Animal | Metabolites |
|---|---|
| Rat (24,30) | 3,5-di-t-butyl-4-hydroxybenzoic acid and its glucuronide |
| | alpha-hydroxy-2,6-di-t-butyl-p-cresol |
| | 3,5-di-t-butyl-4-hydroxybenzaldehyde |
| | 5-(3,5,di-t-butyl-4-hydroxy-benzyl)-N-acetylcysteine |
| Rabbit (1,7) | 3,5-di-t-butyl-4-hydrobenzoyl-beta-D-glucosiduronic acid |
| | beta-(3-t-butyl-2-hydroxy-5-methylethyl)-beta, beta-di-methylethyl-beta-D-glucosiduronic acid |
| | 3,5-di-t-butyl-4-hydroxyhippuric acid |
| Man (9,23) | 3-(1-carboxyl-1-methylethyl)-5-(1-formyl-1-methylethyl)-4-hydroxybenzoyl-beta-D-glucosiduronic acid |
| | 3,5-di-t-butyl-4-hydroxybenzoic acid and its ester glucuronide |

The excretion and elimination of BHT and its metabolites vary greatly with the species. However, in all cases, the elimination of the BHT metabolites appears to be biphasic. The major proportion of the metabolites is eliminated relatively rapidly, the remainder at a slower rate. And in all cases, most of the metabolites are excreted into the bile and are eliminated through the urine and the feces.

In the rat, the metabolites of BHT are excreted rapidly into the bile (95% of a single intravenous dose in 6 hrs.), and relatively slowly into the urine and feces (80-90% in four days). This delay can be attributed to an enterohepatic circulation of one or more metabolites. About 75% of the metabolites are excreted through the feces. Overall excretion is slightly faster in the female rate than in the male, and a larger proportion of the dose is excreted in the urine of the female (40%, vs. 25% in the male), At the termination of a feeding period, the half-life of the BHT which has been deposited in the body fat is 7-10 days (8, 29).

Incomplete studies on rabbits show rabbits eliminate about 2/3 of large single or repeated doses fairly rapidly in the urine. Unchanged BHT has been isolated from rabbit feces, but not from the urine (1, 7).

In man, about three-fourths of a single oral dose (40 mg) is execreted in the urine, with the major proportion appearing within 24 hrs. of dosing. The remainder of the dose is excreted, more slowly, in the feces. There is some controversy as to whether this slow phase is the result of an enterohepatic circulation or release from tissue storage of BHT and its metabolites. There is no conclusive evidence yet, but the general inclination is toward release from tissue storage. The BHT excreted during this slow phase has been found to have a biological half-life of 5 days. All traces of BHT and its metabolites are eliminated within 100 days of termination of dosing (9, 10, 22, 23).

## IV. Effects on Enzymes and Other Biochemical Parameters

There is considerable controversy as to the biochemical effects of BHT. Many researchers feel that BHT directly causes a number of changes in the exposed organism, while others contend that the changes are not a result of the BHT, but rather of various other parameters of the experiments performed (particularly the percentage of fat in the diet and the reduced feed intake resulting from reduced palatability of the BHT-containing food).

For six months, Karplyuk (27) gave rats 4 mg BHT/kg body wt/day in their diet and then 8 mg BHT/kg body wt/day for a second six months. The study covered three generations of rats, all of which were given the indicated levels of BHT. Extensive tests on the treated rats showed no effects of the BHT administered.

In rats at 3000 ppm, a reduction (10-20%) in growth rate of parents and offspring was observed. At this level, an elevation of serum cholesterol was noted after 28 weeks (none after 10wks) and a 10-20% weight increase of the liver after 42 weeks (15). (See biological section for procedural data)

In a study in which 45 pairs of male weanling rats (Norway Hooded) were fed 0.001% BHT in their diet for 5 weeks no changes in any serum constituents were observed. At 0.5% BHT produced increases in the serum cholesterol level, within 5 weeks. In a further study on female rats, 0.1% BHT in a 10% lard diet for 8 months increased the serum cholesterol levels 0.5% BHT in a 10% lard diet for 8 months produced increases in the serum cholesterol, phospholipid and mucoprotein levels (11).

Groups of male Wistar albino rats given 0.01, 0.05, 0.10, and 0.25% BHT in their diet for 12 days showed no difference in body weight compared with the control group. However, the liver weight in relation to body weight was significantly increased at all dietary levels. The activity of microsomal biphenyl 4-hydroxylase was also significantly increased at all levels except 0.01%. In groups of rats fed high fat diets (20% lard or corn oil) for 12 days, significant increases in biphenyl 4-hydroxylase activity were observed. These increases were no greater when 0.01% BHT was incorporated into the diet. The high fat diet, with or without BHT, did not cause an increase in the liver weight compared to the body weight (6).

In rats, after two daily oral doses of BHT (500 mg/kg) significant increases were observed in relative liver weight, aminopyrine demethylase, hexobarbitone oxidase, nitroanisole demethylase and urinary ascorbic acid and urinary ascorbic acid. Continued administration of BHT (up to 21 days) led to continued increases in enzyme activity and urinary ascorbate. When female rats were treated with BHT for one week, the threshold dose for increases in relative liver weight, amino pyrine demethylese, and mexobarbitone oxidase lay between 25 and 75 mg/kg/day (18).

Thirty-six Sprague-Dawley rats were given BHT orally at the rate of 1.7 mg/dietary calorie the first day, 2.2 mg/cal the second day, and 2.8 mg/cal thereafter up to 24 days. The BHT was observed to cause significant hepatomegaly associated with marked proliferation of agranular endoplasmic reticulum, perinucleolar heterochromatin aggregation, and enhanced mitotic activity (31).

Botham, et.al., fed Alderly Park SPF Wistar rats 5, 50, 500, and 2500, mg/kg BHT in maize oil for periods of 7, 10, or 28 days and thereafter on a BHT-free diet for a recovery period of 2-35 days. At various times intervals test and control animals were sacrificed and microscopic studies and enzymatic assays were run. At 5mg/kg, no enzyme response could be detected and no proliferation of the smooth endeplasmic reticulum (SER) could be measured. Botham considers the 50 mg BHT/kg level to be a "threshold" value in that this may be at (or near) the level of SER stimulation in the rat. SER proliferation is closely related to the increased activity of aminopyrine demethylase (3).

Nievel performed several experiments with female Wistar rats. In one experiment, rats were given 220 mg BHT/kg in arachis oil by intubulation for 1-7 days and sacrificed. In another experiment, dosage procedure was identical except for BHT levels of 15, 50, 150, 450, and 800 mg BHT/kg/day. The capacity of rat liver microsomes to incorporate labelled amino acids was depressed at 15 mg/kg and increased up to 450 mg/kg.

At 800 mg/kg the level of incorporation was still increased but less dramatically. Hepatomegaly associated with BHT ingestion results from an increase in the smooth endoplasmic reticulum and increased nucleoli and is not necessarily indicative of toxicity of BHT (35).

Female specific pathogen free Alderly Park rats (Wistar derived) were given 0.01, 0.1, 1.0 and 5% BHT in their diet for periods of 7-10 days. There was a prompt and rapid increase in SER proliferation in response to the 0.1, 1.0 and 5% BHT levels in the diet. Upon cessation of the dietary BHT, the amount of SER fell off rapidly, except in the case of the 5% level where a much more gradual decline was observed. When rats were given 0.01, 0.1 and 1.0% BHT in their diet for 30 days, at the 1% level there was a large progressive increase of SER proliferation. After withdrawal of BHT, there was a marked reduction of the SER, returning to normal within 15 days. In rats given 1% BHT in their diet for 10 days, allowed a 20 day recovery period, then given a subsequent feeding for a further 10 days the SER response to the second feeding was prompt and of the same magnitude of that observed with the initial feeding, suggesting that no change in the rat's ability to deal with BHT had been induced (5).

Feuer, et.al., working with groups of Porton SPF rats found that, when fed BHT in olive oil by intubation at 50, 100, 200, and 500 mg/kg daily for 7 days, rats relative liver weight was increased significantly at 100, 200, 500mg BHT/kg levels. However, the activity of glucose-6-phosphatase was depressed significantly only at 500 mg BHT/kg, and glucose-6-phospate dehydrogenase was exalted significantly only at 500 mg BHT/kg (12).

Gilbert and Goldberg fed Carworth SPF rats 500 mg/kg/day BHT in arachis oil by intubation. They observed the induction of an enzyme, BHT oxidase. At the dosage in the experiment (500 mg BHT/kg/day) the activities of the following enzymes were markedly increased: aniline hydroxylase; hexobarbitone oxidase; BHT hydroxylase; nitroanisole O-demethylase; and aminopyrine N-demethylase. A figure is given, based on in vitro studies, that the metabolic potential for rats is 42 mg BHT/ kg/day (20).

In both male and female rats (Carworth Farm SPF) which were given, by oral intubation, 125 or 500 mg BHT kg/day an increase in relative liver weight and in liver processing enzyme activity was observed. In a further study, rats given 500 mg BHT/kg/day for 1, 2, 3, 4, 7, 14, and 21 days showed a significant increase in hexobarbitone oxidase after 1 day and in relative liver weight, nitroanisole demethylase and amino pyrine demethlase after 2 days. By day 4, relative liver weight and enzyme activity had reached a maximum. In another experiment in which female rats were given doses of 10, 25, 75, 200 and 500 mg BHT/kg/day for one week the threshold dose for liver enlargement and for increased activity of aminopyrine demethylase and hexobarbitane oxydase was determined to be between 25 and 75 mg BHT/kg/day for 7 days (19).

Gaunt, et al., found that rats given 50 mg BHT/kg/day showed increases in liver and adrenal weight and in urinary ascorbic acid excretion (males at 4, 6, 16 wks; females at 4, 12 wks). Histochemical and biochemical examinations showed no significant effect of the BHT upon acid phosphatase, glucose 6-phosphatase, and glucose-6-phosphate dehydrogenase (16). (see biological section for procedural data).

In another study by Gaunt, et. al, rats given 500 mg BHT/kg/day for 14 days showed an increase in relative liver weight, but no change in the ascorbic acid content of the adrenals. BHT stimulated the activity of aminopyrine demethylase, nitroanisole demethylase and hexobartitone oxidose. Liver glucose-6-phosphate was severly depressed but returned to control levels after the recovery period. Glucose-6-phosphate dehydrogenase was unaffected. BHT-treated groups had greatly increased serum cholesterol and phospholipid levels (17). (see biological section for experiment procedure.

Adult rats (six male and six female in each group), which had been reared from weaning on diets that were or were not supplemental with 20% of BHT-free lard or of lard containing 0.5% dietary BHT, were injected with acetate-1-(14)C (20 micro-curies/ 100g body wt) to study the effect of BHT on lipid metabolism. The rats were sacrificed either 1 hr or 3 hr after injection, and the expired $CO_2$ and the lipids from various tissues were isolated and chemically and radiochemically estimated. It was observed that BHT, with or without the dietary lard, increased

the basal metabolic rate, the $(14)CO_2$ expired during the first hour after injection, the concentration of body cholesterol and the rate of synthesis of body and liver cholesterol, and reduced the total fatty acid content of the body. In the rats fed BHT without lard, an increase in the rate of synthesis and turnover of body and liver fatty acids and a reduction in the growth rate were observed. In the rats fed BHT with lard, BHT reduced the rate of synthesis of body and liver fatty acids and reduced the growth rate to a greater extent than in the animals without dietary lard. The authors explained these results by postulating that BHT increases the availability of acetyl coenzyme A and the catabolism of fatty acids (25).

Ames,et.al., working with female rats on vitamin E-free diets, found that BHT at 156 mg/kg lowered litter efficiency to 91 (control 100) and that at 775 mg BHT/kg there was a drastic weight loss in pregnant females with resultant fetal deaths (2).

In a study in which white leghorn pullets were given 0.125% BHT in their feed for a period of one year, the vitamin A and carotenoid pigment content of the eggs, liver and blood serum of the treated birds was observed to be higher than that of the controls (36).

In female White Leghorn chicks fed BHT at 500 mg/kg and or 5000 mg/kg for 3 wks, a reticulocytosis was observed at both dosage levels significantly higher than that of the control group. The worker postulated that the reticulocytosis resulted from the BHT-interference with the necessary oxidation of lipid

In monolayer cultures of rhesus monkey kidney cells, BHT (30 ppm) caused inhibition of RNA and DNA synthesis, thereby causing inhibition of any increase in cell numbers. This effect varies according to sex, with BHT having a greater effect on cells from females. This difference may be the result of more rapid metabolism of BHT in male cells (33).

Very little testing of BHT in man has been done. Attempts to demonstrate induction of a BHT oxidizing enzyme in humans, even at levels well in excess of those encountered in use, have been unsuccessful. It appears that prolonged exposure to BHT does not result in alterations in its handling in man (34)..

V. Drug Interaction

To determine the effect of BHT on fat metabolism, Karplyuk fed male rats 4 mg BHT/kg body weight for 30-35 days. The feeding of fats containning BHT was found to result in a lowering of the iodine number (which indicates the degree of unsaturation of fat) of the liver fats, indicating that BHT inhibits the oxidation of fatty acids in the animal (28).

## VI. Consumer Exposure Information

In 1964, the FDA reported that the intake of BHT for citizens of the United States was 2 mg/day. A 1967 estimate of the consumption of BHT by citizens of the United Kingdom was 1 mg/day (21). A 1961 estimate of BHT consumption by citizens of the U.S.S.R was 2-3 mg/day (22).

Although the conditional acceptable daily intake of BHT set by the Joint FAO/WHO Expert Committee on Food Additives in 1967 is 2 mg/kg body wt./day (26), the provisions of national legislation make the upper limit of intake, assuming that full advantage is taken of all legal tolerances, 4 mg/day/person (19).

The foods in which BHT is found at the highest levels are vegetable oils, animal fats and oils, and foods containing high levels of fats and oils. The level of BHT added to foods is limited to 200 ppm by weight of BHT based on the fat or oil content of the food. BHT has been cleared for use in some specific applications where addition is made based on the total weight of the food (dry breakfast cereals - 50 ppm; potato flakes - 50 ppm; enriched rice - 33 ppm; chewing gum base - 1000 ppm). If other antioxidants are present in the food, then the total level of antitoxidants allowed is the same as that for BHT alone (37).

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Chem. Pharm. Bull. 10: 101-105, 1962



UDC 581.13 : 547.563 : 543.544.42

# 18. Masuo Akagi and Isamu Aoki: Studies on Food Additives. VI.* Metabolism of 2,6-Di-*tert*-butyl-*p*-cresol (BHT) in a Rabbit. (1). Determination and Paper Chromatography of a Metabolite.

(*Faculty of Pharmaceutical Sciences, School of Medicine, Hokkaido University***)

The recent increasing use of food additives is of importance in respect to their acute and chronic toxicity which may be expected when these additives are consumed continuously for a long time. In order to know the actual feature of chronic toxicity of these food additives, their metabolic fate should be examined.

In the course of previous studies on the antioxidative effect of sulfur-containing compounds on concentrated solution of methyl oleate and vitamin A,[1] problems on the metabolic fate of antioxidants in animal body seemed of interest.

Among the important commercial antioxidants added in edible fats, the metabolism of 2,6-di-*tert*-butyl-*p*-cresol (BHT) has never been examined, in contrast to those of butylated hydroxyanisole (BHA), propyl gallate, and 2,4,5-trihydroxybutyrophenone, which were studied by Dacre, *et al.*,[2] Astill, *et al.*,[3] Booth, *et al.*,[4] and Astill, *et al.*[5]

The present investigation is concerned with the determination and paper chromatography of BHT as the preliminary work on its metabolism. A discussion on BHT metabolism is also included.

## Experimental

**Animal, Diet, and Dosage**——The rabbits used weighed 1.8~3.0 kg. They were fed with a standard diet (oats 50 g., carrot 100 g., and cabbage 200 g.) and were kept separately in metabolism cages designed to permit separate collection of urine and feces. BHT filled in a capsule was administered orally in a dose of 0.5 g./kg. body wt. Urinary collection was made every 24 hr. after administration of the compound. The decomposition of metabolites was prevented by the addition of toluene to the collection bottles.

**Materials**——BHT (I) was obtained from a commercial product. 3,5-Di-*tert*-butyl-4-hydroxybenzaldehyde (II), m.p. 1[illegible]°, was prepared by oxidation of BHT with $Br_2$ after the method of Fujisaki,[6] and α-hydroxy-2,6-di-*tert*-butyl-*p*-cresol (III), m.p. 137~138°, by reduction of (II) with $NaBH_4$ in 90% yield according to the modified method of Coppinger, *et al.*[7] 2-*tert*-Butyl-*p*-cresol (IV), m.p. 52°, was synthesized by *tert*-butylation of *p*-cresol with *tert*-BuOH according to Chichibabin's procedure[8] and 3,5-di-*tert*-butyl-4-hydroxybenzoic acid (V), m.p. 210~211°, by the Canizzaro reaction of (II) after the method of Yohe, *et al.*[9] 3,5-Di-*tert*-butyl-4-hydroxybenzylacetate (VI), m.p. 93~99°, was prepared by oxidation of BHT with $Br_2$ in AcOH according to the method of Coppinger, *et al.*[7] α-Methoxy-2,6-di-*tert*-butyl-*p*-cresol (VII), m.p. 100~101°, was synthesized by oxidation of BHT (4 g.) with $Br_2$ (1.2 cc.) in MeOH (20 cc.) at 65° and recrystallized from MeOH-$H_2O$ to blunt needles. Yield, 3.5 g. *Anal.* Calcd. for $C_{16}H_{26}O_2$: C, 76.75; H, 10.47. Found: C, 76.60; H, 10.82.

This compound showed no depression of melting point on admixture with the authentic compound, prepared by treatment of α-bromo-2,6-di-*tert*-butyl-*p*-cresol with MeOH.[9]

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** Kita-12-jo, Nishi-5-chome, Sapporo, Hokkaido ([illegible], 青木 勇).

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4,4'-Ethylenebis(2,6-di-*tert*-butylphenol) (VIII) was prepared by oxidation of BHT with alkaline $K_3Fe(CN)_6$ by the method described by Cook, *et al.*[10] and by Fujisaki.[9]

**Analytical Method**——All spectrophotometric measurements were made by Shimadzu Spectrophotometer Model UV-10.

**Free and Total Phenols**——a) Folin-Ciocalteu Method[11]: This method was useful for the determination of phenol in normal urine and water-soluble phenols, but not for BHT and its derivatives, probably because of the insolubility of the latter in water.

b) Mahon-Chapman method[12]: The calibration curve for BHT satisfied Beer's law in a concentration range of 12~[illegible]γ/cc. with good reproducibility. A satisfactory result was also obtained in recovery test with rabbit urine mixed with a known amount of BHT.

**Glucuronides**——Total glucuronic acid was measured by the naphthoresorcinol picrate method modified by Ishidate.[13] Urine was diluted to give a concentration of 10~[illegible]γ/cc. for glucuronide and readings were made at 570 mμ.

**Ethereal Sulfates**——These were determined by Fiske's modification[14] of benzidine method.

**Paper Chromatography**——Ascending development was employed, using Toyo Roshi No. 51 chromatography paper.

## Results

**Excretion of Normal Metabolites**——The mean daily excretion by undosed rabbits was, from 205±26 cc. of urine of pH 7.6±0.3: Free phenols, 5.3±2.1 mg.; combined phenols, 53.1±2.0 mg.; total glucuronic acids, 12[illegible]±10 mg.; ethereal sulfates, 17±3 mg.

**Metabolites of BHT**——The analysis of urine after administration of BHT is summarized in Table I and Fig. 1. These results show that about 60% of the administered dose is accounted for in free phenols, ethereal sulfates, and glucuronides.

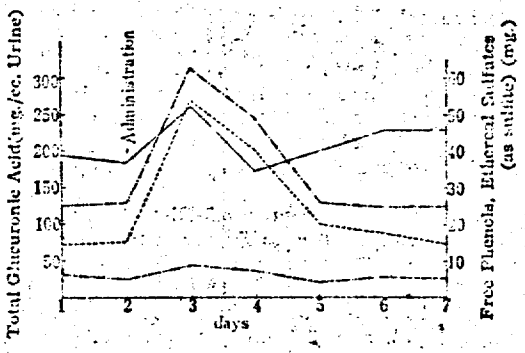


Fig. 1. Quantitative Analysis of the Urine of a Rabbit receiving BHT (1 g. BHT/animal)

——— Urine volume
-·-·-·- Free phenols
- - - - Total glucuronic acid
------ Ethereal sulfates

Results expressed as mean value from six rabbits.

Table I. Metabolites excreted in Rabbit Urine 24 and 48 hours after administration of BHT

| | 24 hr. | 48 hr. | | 24 hr. | 48 hr. |
|---|---|---|---|---|---|
| Urine | yellow (6) | strong yellow | Glucuronides | 24.1±3.5(6) | 13.4±3.6(6) |
| pH | 7.6±0.8(6) | 7.2±0.3(6) | Ethereal sulfates | 10.2±2.3(6) | 6.5±3.1(5) |
| Free phenols | 4.7(2) | 2.1(2) | | | |

Results expressed as mean percentage of the dose with number of experiments in parentheses

**Paper Chromatography of the Derivatives of BHT**——The solvent mixtures (by vol.) used were (A) methanol-water (4:1), (B) petr. ether (b.p. 40~60°)-80% formic acid (2:1), and (C) isopropanol-ammonia-water (20:1:2). The detecting reagents used were (1) Gibbs reagent (0.1% ethanol solution of 2,6-dichloroquinone chlorimide), followed by 5% sodium

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barbital solution; (2) phosphomolybdic acid reagent (0.5% phosphomolybdic acid in ethanol), followed by exposure to ammonia fumes; and (3) diazo reagent (0.3% *p*-nitroaniline in 8% (w/v) hydrochloric acid (25 cc.) mixed with 5% (w/v) of sodium nitrite (1.5 cc.), prepared immediately before spraying. This is followed by addition of sodium carbonate (20%).

The results are shown in Table II. In all cases, the solvent front was run 23 cm.

Table II. Rf Values of BHT Derivatives

| Compound | Rf in solvent system A 1 | A 2[a] | B | C | Coloration to detecting reagents (1) | (2) | (3) |
|---|---|---|---|---|---|---|---|
| (I) Cresol | 0.83 | 0.80 | 0.25 | 0.80 | blue | bluish black | red |
| (II) | 0.84 | 0.56 | 0.15 | 0.89 | " | " | light yellow |
| (I) | 0.93 | 0.11 | 0.95 | | no color | " | no color |
| (III) | 0.88 | 0.77 | 0.43 | 0.92 | violet | yellowish green | yellow |
| (V) | 0.83 | 0.87 | 0.95 | 0.72 | " | " | " |
| (VI) | 0.83 | 0.80 | | 0.75 | " | " | " |
| (II) | | 0.13 | | | no color | bluish black | no color |
| (II) | 0.60 | 0.03 | | 0.96 | " | bluish green | " |

a) Paper (Toyo Roshi No. 51) was sprayed uniformly with camellia oil (10 g.) in $Et_2O$ (100 cc.).

Paper Chromatography of the Metabolites——The solvent used was (C). Compounds were detected on paper chromatograms by spraying (1), (2), or (3) reagent.

The collected 24-hr. urine of rabbit receiving BHT was adjusted to pH 6 with dil. sulfuric acid and continuously extracted with ether for 6 hours. The extract indicated 10 spots (Rf 0.92 ($X_1$), 0.76 ($X_2$), 0.55 ($X_3$), 0.41 ($X_4$), 0.36 ($X_5$), 0.24 ($X_6$), 0.13 ($X_7$), 0.07 ($X_8$), 0.03 ($X_9$), 0.02 ($X_{10}$)). The spots $X_1$, $X_2$, $X_4$, and $X_7$ gave a positive reaction to phosphomolybdic acid reagent, and $X_1$, $X_2$, $X_3$, $X_5$, $X_6$, and $X_8$ were positive to diazo reagent. Among these spots, $X_3$, $X_5$, $X_6$, $X_9$, and $X_{10}$ seemed to be normal components of undosed urine. The spots $X_1$, $X_2$, and $X_7$ gave violet color by the Gibbs reagent, yellowish green by phosphomolybdic acid reagent, and yellow by diazo reagent. It is, therefore, suggested that these spots are those of BHT-metabolites.

The spot $X_1$ was found to be the same as that of *α*-hydroxy-2,6-di-*tert*-butyl-*p*-cresol (II) by paper chromatography run in a different solvent system and by the ultraviolet absorption spectrum. The spot $X_2$ seems to be that of 3,5-di-*tert*-butyl-4-hydroxybenzoic acid (V).

In a later work,[15] these two compounds were isolated from the urine of a rabbit receiving BHT.

The spots at Rf 0.41 ($X_4$), 0.13 ($X_7$), and 0.07 ($X_8$) are also assumed to be those of some additional metabolites of BHT, which were not characterized. These unknown spots are being examined.

When the chromatogram using Toyo Roshi No. 51, with the solvent (A), was sprayed uniformly with camellia oil (10 g.) in ether (100 cc.), a new spot appeared at Rf 0.03, which colored yellowish green to phosphomolybdic acid reagent but not to the Gibbs reagent. This was identical with the spot of synthesized 4,4'-ethylenebis(2,6-di-*tert*-butylphenol) (VI). This compound was later isolated from the BHT-dosed urine.[15]

Unchanged BHT was not detected by paper chromatography in the BHT-dosed urine.

The extract obtained from the feces of rabbit receiving BHT was continuously extracted with ether for 6 hours. By paper chromatography run by the solvent (A, 2), it gave a spot at Rf 0.11 which colored dark blue with phosphomolybdic acid reagent and

15) I. Ando: This Bulletin, 10, 105 (1962).

this was identified with BHT. Further, α-hydroxy-2,6-di-*tert*-butyl-*p*-cresol (IV) was detected in the same extract.

**Isolation of Unchanged BHT in the Feces of Rabbits receiving BHT**——The collected feces were continuously extracted with ether for 10 hours and the extract was evaporated to dryness. The residue was sublimed in a reduced pressure (water pump, 10~15 mm. $Hg$, bath temp., 80°) and the sublimate was recrystallized twice from methanol-water to 0.01 g. of square crystals, m.p. 69~70°. This compound showed no depression of melting point with authentic sample of BHT and had the same ultraviolet and infrared absorption spectra.

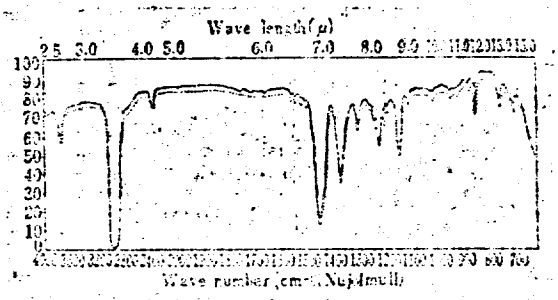


Fig. 2. Infrared Absorption Spectra of Authentic BHT and Isolated BHT

——— authentic BHT
------ isolated BHT

## Discussion

In connection with metabolism of BHT analogs, *p*-cresol was studied by Bray, *et al.*[16] and *tert*-butylbenzene by Williams, *et al.*[17] and by Bernhard, *et al.*[18]. The metabolism of other various phenols has been studied (Williams[19]) and the principal fate of these compounds (ethereal sulfate and glucuronide conjugation, oxidation of alkyl groups) is well known, but less information is available about the metabolism of hindered phenols. BHT is a typical hindered phenol, insoluble in alkaline media, and its hydroxyl group does not react with diazomethane or acetylating agents. An attempt to synthesize the ethereal sulfate by the methods of Elbs,[20] Baumann,[21] Burkhardt, *et al.*,[22] and of Verley,[23] and on glucuronide by the methods of Helferich, *et al.*[24] and Bollenback, *et al.*[25] were also unsuccessful. Thus, BHT behaved in an anomalous manner in chemical reaction, probably because of steric hindrance resulting from two *tert*-butyl groups *ortho* to the hydroxyl group. However, as was found in the present investigation, it is interesting that BHT was excreted 17% by way of ethereal sulfate and 37% by way of glucuronide conjugation.

When BHT was administered to a rabbit, excretion of metabolites was comparatively slow and this may be due to the difficult absorption of BHT from the intestine. Isolation of unchanged BHT from the feces can be considered on the same basis.

Ordinary phenolic compounds give a blue color with the Gibbs reagent, but *para*-substituted phenols give no color reaction. It is interesting that α-hydroxy-2,6-di-*tert*-

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butyl-*p*-cresol, 3,5-di-*tert*-butyl-4-hydroxybenzoic acid, and 3,5-di-*tert*-butyl-4-hydroxybenzylalcohol (derivatives of BHT and, *ortho*- and *para*-substituted phenols) were found to give violet color with the Gibbs reagent.

The authors are indebted to Prof. T. Ukita of the University of Tokyo for his kind advice and suggestion, to Mr. Hugo of this Faculty for the determination of glucuronic acid, and to Chugai Pharmaceutical Co. Ltd. for their supply of glucuronolactone. Thanks are also due to Mr. Nerra of the analysis room of this Faculty for the elementary analysis. This work was supported partly by a Grant-in-Aid for Scientific Research provided by the Prefectural Government of Hokkaido to which the authors' thanks are due.

## Summary

Metabolism of the antioxidant, 2,6-di-*tert*-butyl-*p*-cresol (BHT), was studied in a rabbit. After administration of 0.5 g./kg. of BHT by mouth, 37.5% of it was excreted as glucuronides, 16.7% as ethereal sulfates, and 6.8% as free phenols.

α-Hydroxy-2,6-di-*tert*-butyl-*p*-cresol, 3,5-di-*tert*-butyl-4-hydroxybenzoic acid and 4,4'-ethylenebis(2,6-di-*tert*-butylphenol) were identified by paper chromatography from the rabbit urine 24 hours after the administration of 0.5 g./kg. of BHT.

Unchanged BHT was isolated from the feces, but not from the urine.

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# Determination of Butylated Hydroxyanisole, Butylated Hydroxytoluene, and Ethoxyquin in Hydrocarbon-Soluble Samples

N. J. ALICINO, H. C. KLEIN, J. J. QUATTRONE, Jr.,[1] and T. K. CHOY

Fine Chemical Division, Nopco Chemical Co., Harrison, N. J.

A rapid systematic method for the analysis of BHT and BHA in the presence or absence of ethoxyquin, based on solvent extraction and column chromatographic procedures, is described. The sample of food or feed containing the antioxidants is first dissolved in a suitable hydrocarbon. A sample of this solution is thoroughly shaken with 1N HCl. Ethoxyquin if present is found in the aqueous phase which is separated off and determined by ultraviolet spectrophotometry. The solvent phase contains mostly BHA, BHT, and other organic ingredients. The BHA is then brought into a 70% ethanol solution, and determined either colorimetrically or by ultraviolet absorption spectrophotometry. Another sample of the original hydrocarbon solution is passed through a Florisil chromatographic column. All antioxidants except BHT will remain absorbed on the column. The BHT in the eluted solution is then determined by ultraviolet spectrophotometry.

Quantitative determinations of BHA (2- or 3-*tert*-butyl-4-hydroxyanisole) and BHT (3,5-di-*tert*-butyl-4-hydroxytoluene), when present together and in mixtures along with ethoxyquin (1,2-dihydro-6-ethoxy-2,2,4-trimethyl quinoline), pose a difficult analytical problem because of the interference of these antioxidants with each other and with other chemical constituents usually found in complicated food and feed formulations. Although there are many methods cited in the literature (*1–10*) for the determination of these antioxidants, the procedures, in general, are long and tedious, do not permit the separate determination of these compounds when present together, and are usually only applicable for a specific case.

The object of this paper is to describe a systematic procedure for the determination of BHA, BHT, and ethoxyquin when present alone or in combination in hydrocarbon-soluble samples. (The procedure for the determination of these antioxidants when present in water-soluble samples is at present in preparation.) This procedure employs, with necessary modification, several of the methods already described in the literature along with several innovations. In arriving at this procedure, speed as well as accuracy has been the object of primary consideration.

## Reagents

The reagents used as solvents for extraction of BHT were first purified by passing them through a Florisil column and discarding the first two 25-ml. portions effluxed. The ultraviolet absorptions of these solvents were then checked.

Isoöctane, redistilled, reagent grade.

Petroleum ether, b.p. 40° to 60° C.

Skellysolve B, redistilled.

BHA, twice recrystallized from petroleum ether, m.p. 59° to 60° C.

BHT, (Nopco Food Grade). Purity 99% (min.), m.p. 69° to 70° C.

Ethoxyquin, (freshly prepared by the authors' research laboratory for an arbitrary standard) and commercial samples supplied from two sources.

## Apparatus

Spectrophotometer, Beckman ultraviolet model DU.

Colorimeter, Beckman DU model B or Evelyn colorimeter.

## Materials

Glass wool.

Florex XXS (calcinated), 60- to 100-mesh, obtained from Floridin Co. The water content of each 1-pound batch of this material is approximately 6 to 7% as determined by the manufacturer.

Florisil, 60- to 100-mesh, Floridin Co., Tallahassee, Fla.

## Preparation of Columns

Into a 25-cm. long, 20-mm. diameter chromatographic column, insert a small plug of glass wool and add with gentle tapping about 20 grams of Florisil which has been prewashed with the solvent before use. Then wash with two 15-ml. portions of solvent.

## Experimental

A solid or liquid sample, containing BHA, BHT, and ethoxyquin weighed to the nearest 0.15 mg., is dissolved in 100 ml. of heptane. Other hydrocarbons, such as hexane (redistilled, reagent grade), cyclohexane (redistilled, reagent grade), isoöctane, Skellysolve B, and petroleum ether, can also be used in place of heptane. These solvents have been tried and were found to give similar results. The sample hydrocarbon mixture is shaken for a few minutes to ensure that the sample is completely dissolved into the hydrocarbon.

A 25.0-ml. sample of this hydrocarbon solution is then passed through the Florisil-packed chromatographic column. All antioxidants and other interfering substances with the exception of BHT are retained on the column. Additional solvent is passed through the column so that the eluent will total about 180 ml. The eluate is collected in a 200-ml. volumetric flask and diluted to this volume with solvent. BHT can then be determined from this solution. The ultraviolet absorbance is read at 272, 277, 280, 283.5, and 287 mμ; an inverted w-shaped spectrum, as shown in Figure 1, which is a qualitative test specifically known for this antioxidant indicates the presence of BHT. A linear relationship is shown in Figure 2 of absorbance *vs.* BHT concentration over the range from 0.02 to 0.12 mg. per ml. (corr.). Although either of these wavelengths can be used, calculations using the absorbance at 283.5 mμ were used.

The weight of sample taken depends on its antioxidant content. This method gives good results when about 0.05 mg. per ml. of BHT is present in the sample. The amount of BHA can be as little as half of the amount of BHT in the sample; however, further dilution of the

[1] Present address: Physics Department, Fairleigh Dickenson University, Teaneck, N. J.

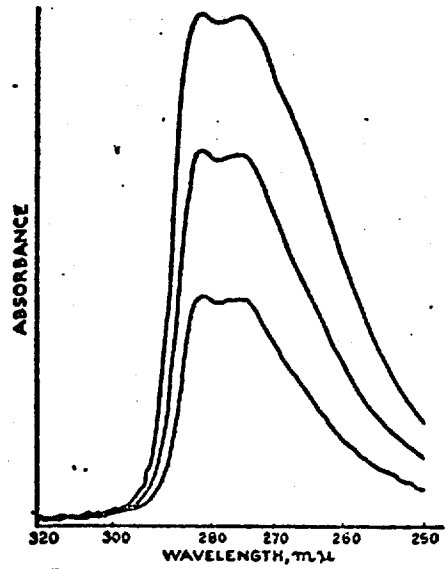


Figure 1. Absorbance vs. wavelength of various concentrations of BHT

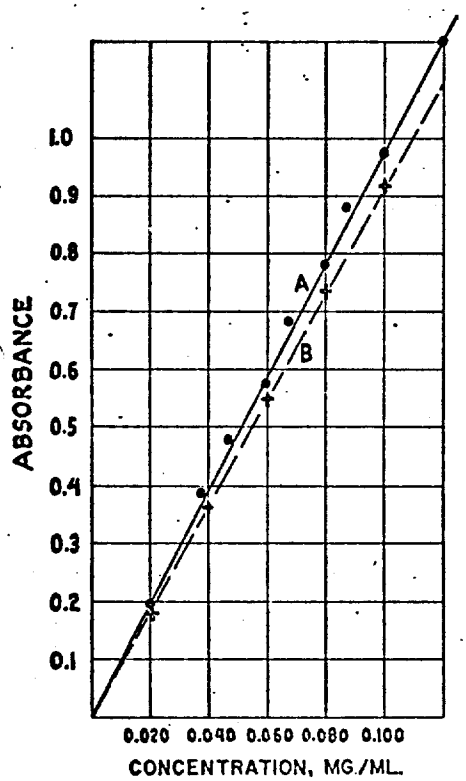


Figure 2. Absorbance at 283.5 mμ (A) and 277 mμ (B) vs. concentration of BHT known solution

alcohol extract may be used for the ultraviolet absorption readings.

A 35-ml. sample of the heptane solution is used for the determination of ethoxyquin and BHA. The heptane solution is first shaken with about 15 ml. of 1*N* HCl in a 100-ml. separatory funnel. The ethoxyquin present in the sample is retained in the HCl phase, which is separated and used for the determination of ethoxyquin by the ultraviolet spectrophotometric method previously described (*4*).

To ensure that the extraction of the ethoxyquin by the HCl solution is complete, the above procedure is usually repeated three times. The extracts are then combined and used for the determination.

The heptane solution remaining in the separatory funnel is then washed several times with distilled water to remove any residual acid left in this phase. The BHA present is then extracted by treatment with three separate portions of 25 ml. each and a fourth portion of 15 ml of a 70% ethanol solution (USP, 190 proof, 700 ml. of ethanol made up to 1 liter with distilled water) made up to 100.0 ml. with ethanol. The BHA present is then determined spectrophotometrically. A linear plot of BHA concentrations *vs.* absorbance is shown in Figure 3.

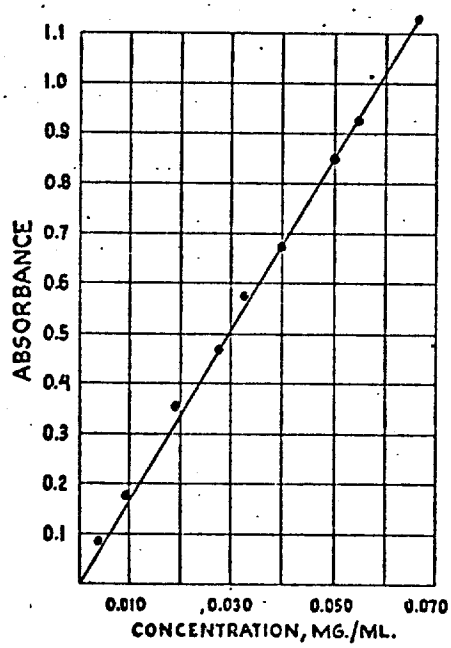


Figure 3. Absorbance at 292 mμ vs. concentration of BHA solution

### Results and Discussion

To ascertain the accuracy of analysis for BHA and BHT together with, and in the absence of, ethoxyquin, triplicate runs were performed on the standard antioxidant solution. The average percent deviation for the determinations of BHA and BHT was found to be ±1.0% for the standard mixture not containing ethoxyquin. When ethoxyquin was present at the same concentration of BHA, the percent accuracy for the determination of BHA was found to be higher than 97%, and no effect was observed on the BHT determination. The results of this study are given in Table I and Table III. The column chromatographic method used here for the separation and determination of BHT is much simpler than that described by Phillips and Hinkel (*9*). The greatest advantage of the Florisil column is that it reduces the time required for complete column separation from 4 to 5 hours to about 25 minutes. Also, the activation and deactivation appear to be less critical for this column than might be expected. The object of this activation-deactivation is to maintain the water content of the column material at about 6% by weight. A number of experiments gave good results with this column even without preliminary treatment. However, if a large amount of vitamin A is present, a previous deactivation of the Florisil column having a 6 to 7% water content is recommended.

Another advantage of this column is that any of the solvents enumerated above can be used for the extraction of the BHT. However, for samples con-

**Table I. Percent Recovery of BHT and BHA in Mixture Samples after Column and Solvent Partition Extraction, Respectively**

| BHT, Mg./Ml. | | | BHA, Mg./Ml. | | |
|---|---|---|---|---|---|
| Actual | Found[a] | Recovery, % | Actual | Found[a] | Recovery, % |
| 0.0050 | 0.0049 | 98.0 | 0.0020 | 0.0019 | 98.5 |
| 0.0080 | 0.0078 | 98.8 | 0.0050 | 0.0049 | 98.0 |
| 0.0100 | 0.0098 | 98.0 | 0.0104 | 0.0103 | 96.7 |
| 0.0200 | 0.0196 | 98.0 | 0.0075 | 0.0073 | 97.3 |
| 0.0502 | 0.0499 | 99.4 | 0.0150 | 0.0145 | 96.7 |
| 0.1050 | 0.1030 | 98.1 | 0.0200 | 0.0193 | 96.5 |
| 0.1500 | 0.1480 | 98.7 | 0.0500 | 0.0493 | 98.6 |

[a] Average of triplicate runs.

**Table II. Determination of BHA and BHT of Known Solutions**

| BHA, Mg./Ml. | | BHT, Mg./Ml. | |
|---|---|---|---|
| Actual | Found | Actual | Found |
| 0.0020 | 0.0019 | 0.0050 | 0.0048 |
| | 0.0020 | | 0.0049 |
| | 0.0019 | | 0.0047 |
| 0.0050 | 0.0048 | 0.0100 | 0.0098 |
| | 0.0049 | | 0.0098 |
| | 0.0047 | | 0.0097 |
| 0.0104 | 0.0104 | 0.0200 | 0.0198 |
| | 0.0102 | | 0.0195 |
| | 0.0103 | | 0.0196 |
| 0.0200 | 0.0193 | 0.0502 | 0.0494 |
| | 0.0196 | | 0.0492 |
| | 0.0194 | | 0.0496 |
| 0.0500 | 0.0492 | 0.1050 | 0.1030 |
| | 0.0494 | | 0.1040 |
| | 0.0493 | | 0.1035 |

**Table III. Determination of BHA, BHT, and Ethoxyquin of Known Solutions**

| BHA, Mg./Ml. | | BHT, Mg./Ml. | | Ethoxyquin, Mg./Ml. | |
|---|---|---|---|---|---|
| Actual | Found | Actual | Found | Actual | Found |
| 0.0025 | 0.0023 | 0.0050 | 0.0048 | 0.0025 | 0.0025 |
| | 0.0022 | | 0.0049 | | 0.0024 |
| | 0.0022 | | 0.0047 | | 0.0024 |
| 0.0050 | 0.0047 | 0.0100 | 0.0098 | 0.0050 | 0.0048 |
| | 0.0048 | | 0.0098 | | 0.0049 |
| | 0.0046 | | 0.0097 | | 0.0047 |
| 0.0150 | 0.0146 | 0.0200 | 0.0198 | 0.0100 | 0.0098 |
| | 0.0145 | | 0.0195 | | 0.0099 |
| | 0.0147 | | 0.0196 | | 0.0097 |
| 0.0250 | 0.0242 | 0.0502 | 0.0494 | 0.0250 | 0.0243 |
| | 0.0243 | | 0.0492 | | 0.0242 |
| | 0.0244 | | 0.0496 | | 0.0245 |
| 0.0500 | 0.0488 | 0.1050 | 0.1030 | 0.0500 | 0.0494 |
| | 0.0490 | | 0.1040 | | 0.0493 |
| | 0.0491 | | 0.1030 | | 0.0492 |

**Table IV. Determination of BHA, BHT, and Ethoxyquin in Commercial Samples**

| Sample* | BHA, Mg. | | BHT, Mg. | | Ethoxyquin, Mg. | |
|---|---|---|---|---|---|---|
| | Theory | Found | Theory | Found | 100% Claim | Found |
| Vit. A, oil A | 4.20 | 4.12 | 4.20 | 4.14 | ... | ... |
| | | 4.10 | | 4.12 | | |
| | | 4.10 | | 4.15 | | |
| oil B | 6.80 | 6.72 | 7.50 | 7.38 | ... | ... |
| | | 6.70 | | 7.42 | | |
| | | 6.68 | | 7.44 | | |
| oil C | 3.50 | 3.38 | 3.50 | 3.42 | ... | ... |
| | | 3.41 | | 3.44 | | |
| | | 3.40 | | 3.44 | | |
| Multiple vit., premix I | 12.00 | 11.88 | 18.00 | 17.75 | ... | ... |
| | | 11.73 | | 17.72 | | |
| | | 11.72 | | 17.78 | | |
| Multiple vit., premix II | 20.00 | 19.64 | 15.00 | 14.85 | 25.00 | 24.62 |
| | | 19.68 | | 14.87 | | 24.65 |
| | | 19.63 | | 14.84 | | 24.67 |
| Multiple vit., premix III | 5.00 | 4.82 | 5.00 | 4.90 | ... | ... |
| | | 4.85 | | 4.88 | | |
| | | 4.86 | | 4.86 | | |
| Nopco Feed, premix L | 7.50 | 7.36 | 7.50 | 7.41 | 3.40 | 3.28 |
| | | 7.32 | | 7.38 | | 3.29 |
| | | 7.35 | | 7.40 | | 3.28 |
| Nopco Feed, premix V | 2.80 | 2.68 | 2.80 | 2.72 | 3.20 | 3.12 |
| | | 2.65 | | 2.72 | | 3.11 |
| | | 2.66 | | 2.70 | | 3.13 |

* The first three samples are vitamin A (palmitate or acetate) products. The vitamin and feed premixes contain all or most of the listed ingredients: vitamins A and D, niacin, vitamin $B_{12}$, *d*-calcium pantothenate, riboflavin, BHT, BHA, and ethoxyquin either in wax or ethyl-cellulose coating.

taining a low concentration of BHT, Skellysolve B or petroleum ether is recommended, since an evaporation procedure at a reduced pressure may be necessary. For common samples, hexane or heptane were found to give good results, and these were the solvents used in obtaining the data presented in this paper.

Florex XXS can also be used effectively as a column filter. Unfortunately, when the column was prepared for some individual batches, the effluents were found to give high ultraviolet absorption readings. This difficulty can be remedied by washing the column thoroughly with solvent before using, but the procedure is tedious.

Table II shows the actual recovery of BHA and BHT from known standard solutions of these two antioxidants when present alone. Table III gives the recovery of BHA, BHT, and ethoxyquin from known solutions of varying concentrations of these antioxidants. When traces of BHT, tocopherol, vitamin additives, and other commonly interfering ingredients were brought into the alcoholic solution, no significant change of the ultraviolet absorption was observed. Thus, this procedure may prove to be specific for the separation and determination of BHA, and can be used to determine this antioxidant in commercial products. This method then would enable one to determine the concentration of BHA directly without the tedious steam distillation process described by Anglin, Mahon, and Chapman (*7*).

Table IV gives the results for the determination of BHA, BHT, and ethoxyquin in a number of commercially available samples. (Samples used for the evaluations were freshly prepared.) Where the concentrations of antioxidants are known, they are indicated in the table. The average percent recovery of BHA from these various samples was found to be 97 ± 2%.


### Acknowledgment

The authors wish to express their thanks to the personnel in the Vitamin Assay Laboratory of Nopco Chemical Co. who performed many of the determinations used in these tables.


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# *Research Section*

## Effects of Butylated Hydroxytoluene on the Enzyme Activity and Ultrastructure of Rat Hepatocytes

CHRISTINE M. BOTHAM, D. M. CONNING, JO HAYES, M. H. LITCHFIELD and T. F. MCELLIGOTT

*Imperial Chemical Industries Limited, Industrial Hygiene Research Laboratories, Alderley Park, Nr. Macclesfield, Cheshire, England*

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**Abstract**—The response of the drug-metabolizing enzyme, amino-pyrine demethylase, to BHT fed to rats at dietary levels of 0–5% for up to 28 days has been compared with the proliferative response of the smooth endoplasmic reticulum (SER) in the liver, as measured by a point-counting technique. The time-course of the responses is comparable, but the enzyme response is the more sensitive. There is evidence, however, that at the lower dose levels, the enzyme response may not always be accompanied by a maintained increase of SER and it is suggested that this may represent the limit of the physiological response. Cessation of BHT administration is followed by a rapid disappearance of the excess SER by a process still undetermined. Subsequent exposure to further dietary BHT results in a response similar to the initial one.

### INTRODUCTION

The administration to rats of many compounds including drugs, food additives, pesticides and carcinogens has been shown to stimulate the activity of hepatic microsomal enzymes associated with drug metabolism, and in many instances this increased activity is accompanied by proliferation of the smooth endoplasmic reticulum (SER) of the hepatic cells (Remmer & Merker, 1963). Oral administration of the antioxidant, butylated hydroxytoluene (BHT), has been shown to cause an increase in the weight of the liver and to increase the activity of five hepatic microsomal enzymes (Gilbert & Golberg, 1965). More recently Lane & Lieber (1967) showed that BHT also caused SER proliferation in rat hepatocytes and was associated with enhanced mitotic activity in these cells.

The present study was undertaken to determine whether SER proliferation in the rat occurred simultaneously with the increased activity of drug-metabolizing enzymes during BHT administration and whether any such association could be placed on a quantitative basis, and, in particular, to establish how long the observed changes persisted after the feeding of BHT was stopped. In further experiments attempts were made to determine whether prior feeding with BHT led to more rapid drug metabolism. It was found that SER proliferation is closely related to the increased activity of aminopyrine demethylase (APDM) both chronologically and quantitatively, and does not persist after cessation of exposure to the compound. Previous exposure does not appear to facilitate subsequent enzyme or ultrastructural changes.

### EXPERIMENTAL

*Animals and diet.* Female rats of the Alderley Park SPF Wistar strain (initial body weight 150–175 g) were housed in groups of five and maintained on a basic diet comprising 80%

(w/w) powdered rat diet (from Messrs. Oakes, Congleton, Cheshire), 2% (w/w) maize oil and 18% (w/w) malt extract. To prepare the test diets, the appropriate amount of BHT was dissolved in the maize oil, the oil being slightly warmed if necessary to effect solution, and the diet was made up in the usual way. Control animals were maintained on diets prepared in the same way, without the inclusion of BHT.

*Experimental design and conduct.* There were two experiments. In the first, groups of rats were maintained on diets containing 0·0, 0·01, 0·1, 1·0 or 5·0% BHT for periods of 7, 10 or 28 days, and were fed thereafter on a diet free of BHT for a recovery period of 2–35 days. Animals were killed at intervals during these periods in groups of four, two being used for enzyme assay and two for electron microscopy, except at days 2, 4 and 7 of the recovery period following feeding with diet containing 1% BHT, when four animals were used for enzyme assay. The animals were killed by cervical dislocation and control animals were killed at the same time.

In the second experiment animals were given 1% BHT for 10 days, and then for a second period of 10 days after an interval of 20 days on a normal diet. These animals were killed in groups of five at 10, 30, 40, 42 and 47 days. Electron-microscopy studies were performed on each animal and enzyme assays on five or three animals of the same groups.

*Enzymes preparation and assay.* APDM was selected as the enzyme for study because the assay is sensitive and convenient to operate and gives low control values. The method relies on the appearance of a reaction product (aminoantipyrine) which makes it more precise than methods dependent on the disappearance of substrates. The homogenizing medium and conditions, and the subsequent assay for APDM were those described by Gilbert & Golberg (1965). Livers were assayed in pairs, being homogenized in glass tubes at 4°C immediately after removal from the animals.

*Tissue preparation.* For electron microscopy, tissues were obtained from pairs of rats at the times indicated and were treated as follows. Immediately after killing, a thin slice of liver was taken from the lower part of the right inferior lobe. The tissue was placed in a drop of chilled fixative on a wax surface, minced rapidly by means of a razor blade and then fixed at 0°C in an excess of 1% buffered osmium tetroxide for 2–3 hr. After fixation the tissues were dehydrated through ascending grades of ethyl alcohol into epoxypropane and embedded in araldite, which was then polymerized for 2 days at 60°C. Sections were cut with an LKB "Ultratome" and stained with uranyl acetate and lead citrate, or with lead citrate alone. Sections were examined in an AEI EM6B electron microscope.

*Quantitative assessment of ultrastructural changes.* Assessment of the ultrastructural changes was placed on a quantitative basis by two methods. Initially, photographs of randomly selected fields at a standard final magnification of 15,000 diameters were divided into centimeter squares by means of a grid which could be laid over the photograph, each grid comprising 420 such squares. Several squares were selected randomly and the approximate area occupied by a given cell constituent within the square was estimated. From a simple total the proportion of constituents one to another could easily be calculated. This method was later replaced by a modified version of the differential point-counting technique (Glagoleff, 1933, cited by Weibel, Kistler & Scherle, 1966) in which photomicrographs at a magnification of 15,000 diameter were overlayed by a grid of 440 points, 1 cm apart. Comparison of the two methods has, in fact, revealed no difference between the results obtained (Jo Hayes, unpublished results, 1968), but the latter system is much easier to operate. The results were usually expressed in terms of units of SER, which were calculated either by relating SER to the total cell constituents or by relating SER to mitochondria, the form of

being generally used. The number of SER points was divided by the number of points on other cell constituents, and the resultant figure was termed the unit of SER. In the first experiment 3–5 micrographs were examined from each animal, and in the second experiment groups of five animals were used and a total of 25 micrographs from each group was analysed.

## RESULTS

### *Enzyme assay*

The response of APDM activity to BHT administration is well established within 7 days (Table 1). The magnitude of the response varies with the dietary level, the lowest level (0·01 %) producing no detectable increase in enzyme activity during the 28-day feeding period. When the animals were returned to normal diet the raised levels of enzyme activity diminished rapidly, so that even after 28 days on a 1 % diet, the regime producing the highest level of enzyme activity recorded, the values had returned to normal by the seventh day on the normal diet.

**Table 1.** *APDM activity following the feeding of 0·01–1 % BHT in the diet for 7–28 days, followed by a recovery period of up to 35 days on basic diet*

| Dietary level of BHT (%) | Feeding of test diet* (days) | APDM activity (μmoles/hr/g liver) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Test period: Activity at day no. | | | | Recovery period: Activity at day no. | | | | | | |
| | | 7 | 10 | 14 | 28 | 2 | 4 | 7 | 14 | 21 | 28 | 35 |
| 0·01 | 10 | | 0·07 | | | | 0·055 | | | | | |
| | 28 | | | 0·09 | 0·10 | 0·07 | | | | | | |
| 0·1 | 10 | | 0·24 | | | | 0·065 | 0·11 | 0·085 | | | |
| | 28 | | | | 0·22 | | 0·165 | | | | | |
| 1·0 | 7 | 0·695 | | | | | | 0·145 | 0·16 | 0·155 | 0·135 | 0·14 |
| | 28 | | | | 0·81 | 0·22† | 0·233† | 0·16† | | | 0·13 | |

* Duration of BHT feeding prior to return to basic diet.
† Values are means of four rats, while in all other cases values are means of two.
Mean ± SD for 42 control rats was 0·10 ± 0·04.

### *Electron microscopy*

The degree of SER proliferation was directly proportional to the amount of BHT in the diet and to the duration of feeding at the 1 and 5 % levels. At the 0·1 % level, the response again appeared to be proportional among those animals fed BHT for 10 days, but when feeding was prolonged, the initial rise disappeared and thereafter values outside the control limits only occurred at the end of the feeding period, and were not, at this time, statistically significant (Fig. 1). No effect on SER was seen at the 0·01 % level.

More prolonged feeding of diet containing 1 % BHT caused a more vigorous response than when a shorter feeding period was used. In the latter case, however, the response persisted for 7 days and, in view of the early response at this dietary level in the prolonged feeding experiment (Fig. 2), it is likely that the low response on the shorter feeding test is [illegible]. In animals receiving 1 % BHT for 28 days, changes could be detected at 2 days [illegible] marked at 3–4 days (Fig. 2), and the highest levels of all were recorded in this group,

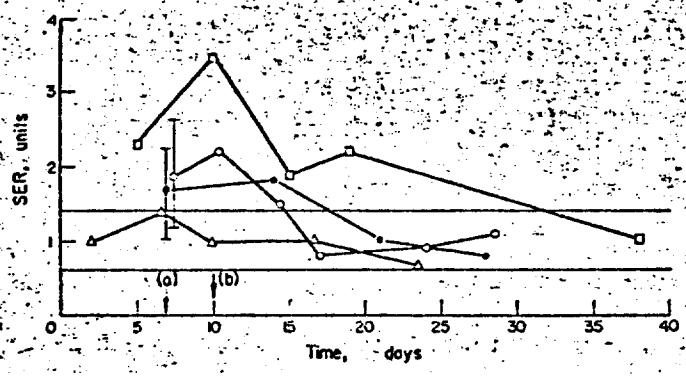


FIG. 1. SER response to feeding of BHT for 7 or 10 days at dietary levels of 0·01% (△), 0·1% (○), 1% (●) and 5% (□). Arrows indicate the end of the feeding period for (a) 1% BHT and (b) 0·01, 0·1 and 5% BHT. Standard deviations are given at relevant points and control limits (1·0 ± 0·4 SD) are indicated by horizontal lines.

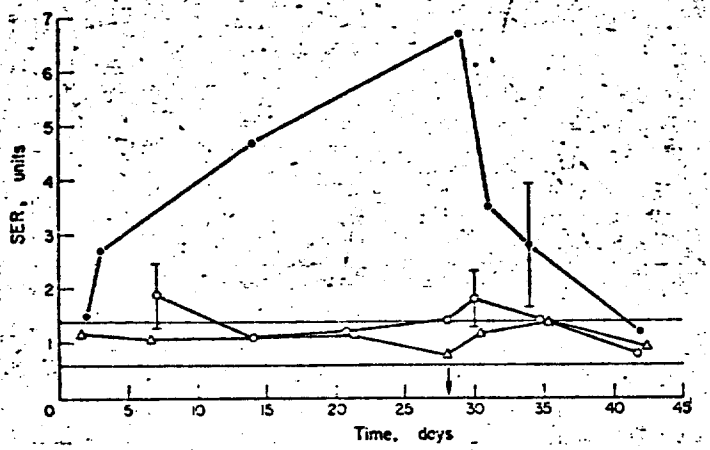


FIG. 2. SER response to feeding of BHT for 28 days at dietary levels of 0·01% (△), 0·1% (○) and 1% (●). An arrow marks the end of the feeding period. Standard deviations are given at relevant points and control limits (1·0 ± 0·4 SD) are indicated by horizontal lines.

reaching a peak of 6·8 at 30 days (i.e. 2 days after cessation of BHT feeding). No estimation has been made of the number of cells involved, but many hepatic cells showed large areas of the cytoplasm given over to fairly tightly packed SER, much of it markedly convoluted. Because of the convolutions the occurrence of branching of tubules was difficult to determine, but this occasionally occurred (Fig. 3). In unstarved animals the proliferated SER was associated with glycogen, but generally the animals were starved overnight before death and the consequent absence of glycogen allowed easier identification of the SER. The other cytoplasmic contents showed little abnormality except that the mitochondria contained more dense 'microbodies' than usual and the bounding membrane appeared somewhat crenellated. Rough endoplasmic reticulum (RER) could be detected among the SER, but formed a very minor proportion of the total. The nuclei showed prominent nucleoli, occasionally two in number, but were otherwise normal. There was no increase in lysosomal activity, but towards the later stages of feeding, fatty vacuolation became more obvious.

In summary, therefore, quantitative assessments revealed very marked increases in SER proliferation at the 1 and 5% levels. At the 0·1% level, measurements of SER showed a temporary rise initially and thereafter did not exceed the normal until the feeding period had ended, though there had been a gradual rise throughout that period. The 0·01% dietary level provoked no proliferation. Following cessation of BHT administration there was fairly rapid dissolution of the proliferated SER. The SER membrane became blurred in outline and stained less and less well, so that eventually all that remained was the space where the tubules had been together with a few tubules of RER and such SER that survived (Fig. 4). This all occurred within 8 or 9 days of cessation of feeding at the 1% level, but took longer after 5% feeding. There was evidence of an increase of polysomes and RER, and occasional cells showed increased fatty vacuolation.

The experiments designed to show whether liver previously subjected to BHT was better able to handle subsequent administration, showed that the enzyme activity did not differ significantly following a 10-day period of administration of 1% BHT 20 days after the cessation of a similar period of administration (Table 2). Electron microscopy also showed that the magnitude of SER response was virtually identical during both periods of administration, and that the disappearance of the proliferated SER was as rapid as that observed in the previous experiment (Fig. 5).

## DISCUSSION

The response of APDM activity and SER to BHT administration did not differ in this experiment from that reported previously using fairly high dietary levels (about 1%) of BHT (Lane & Lieber, 1967). The degree of the SER response has been shown to be proportional to the amount of BHT in the diet, and, therefore, to correlate well with the enzyme activity which is also increased in proportion to the greater stimulus. This is at least circumstantial evidence that the enzyme activity is contained in the SER. This interpretation is strengthened by the fact that, at the 0·01% level, no enzyme response could be detected and no proliferation of SER could be measured. A curious effect was noted at the 0·1% level, in that there was a moderate rise in enzyme activity associated with a transient rise

Table 2. *APDM activity in response to two 10-day periods of feeding of 1% BHT in the diet interrupted by a 20-day recovery period on basic diet*

| APDM activity (μmiles/hr/g liver) | | | | |
|---|---|---|---|---|
| | | | After subsequent consumption of basic diet for | |
| After 10 days on 1% BHT | After 20-day recovery period | After second 10-day period on 1% BHT | 2 Days | 7 Days |
| 0·55 | 0·09 | 0·70 | 0·32 | 0·12 |
| 0·97 | 0·09 | 0·67 | 0·29 | 0·17 |
| 0·68 | 0·08 | 0·53 | 0·38 | 0·19 |
| 0·86 | | 0·70 | | |
| 0·91 | | 0·58 | | |
| Mean ± SD 0·79±0·18 | 0·09 | 0·64±0·07 | 0·33 | 0·16 |

Values are for individual rats. Mean ± SD for three control rats was 0·08 ± 0·04.

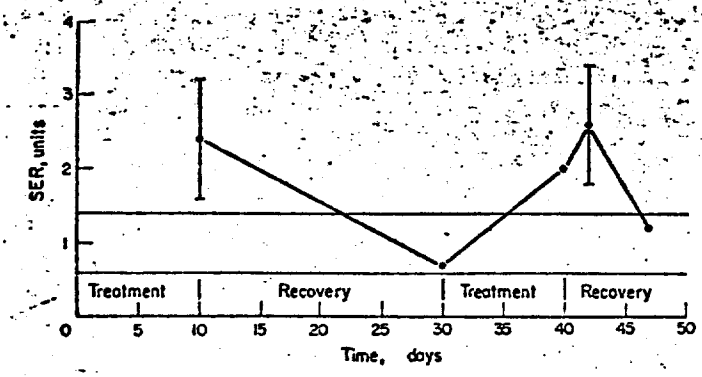


FIG. 5. SER response after repeated episodic administration of BHT at a dietary level of 1%. Standard deviations are given at relevant points and control limits (1·0 ± 0·4 SD) are indicated by horizontal lines.

of SER units during a 10-day period of feeding and during the early part of a 28-day period, and in the latter test group these effects recurred after feeding had ceased. It is possible that these early changes represent an immediate SER response, which is no longer required once metabolic activity is established. This dosage level may thus be near the 'threshold' level at which SER production may be stimulated.

An unexpected finding was the rapid disappearance of SER following cessation of BHT feeding. This largely disappeared in 6 days following prolonged feeding, and disappeared entirely in 14 days. Following shorter periods of feeding of 5% BHT the proliferated SER took longer to resolve, but the process was completed in 28 days. It is remarkable that the removal of relatively large amounts of lipid membranes should be effected so quickly.

The means of removal of the proliferated membranes is obscure in that there was no evidence of the increased lysosomal activity or formation of autophagocytic vacuoles that might have been expected. The membranes merely developed an indistinct outline, showed reduced ability to be stained and eventually merged with the background cytoplasm. Disappearance of the SER left large areas of unstained cytoplasm, but the total amount of SER in these circumstances rarely fell below the control levels. During the later stages of the recovery period, however, there was an apparent increase of RER and polysome production, which may have been associated with the replenishment of SER. Although not observed on this occasion, mitotic activity has been seen in livers of animals treated with BHT (Lane & Lieber, 1967). This reaction of the liver, and proliferation of RER may come into the category of tissue reactions to injury. Increased numbers of mitotic figures have been observed in the liver after the administration of liver poisons (Popper & Schaffner, 1957) and proliferation of RER is an early accompaniment of cell repair.

In some reported studies, proliferated SER has been associated with the production of myelin figures (Herdson, Garvin & Jennings, 1964) of the type associated with toxic reactions or carcinogens (Emmelot & Benedetti, 1960); and it has been suggested that these represent degeneration of SER (Burger & Herdson, 1966). As has been stated, none of the conventional evidence of lipid-membrane degeneration (e.g. myelin figures, cytosome formation and lysosomal activation) occurred in the present experiments even during the rapid phase of SER removal. Lane & Lieber (1965) also failed to detect these "figures" after BHT administration. In experiments where they have been described there is usually evidence of mitochondrial damage and reduction in the numbers of mitochondria. Although the

mitochondria are altered after high doses of BHT, the numbers are not reduced. It may be, therefore, that myelin figures are associated with mitochondrial damage, and not with damaged SER. There is evidence to this effect in pulmonary and renal cells under the influence of cytotoxic substances (D. M. Conning, unpublished data, 1969).

The control of SER production is not known, and hence the toxicological significance remains obscure. It is known that apparently innocuous materials can cause proliferation as well as more dangerous materials. One may instance softwood shavings used as bedding (Vesell, 1967) and various foodstuffs. It is likely that initially the response is physiological, but the limit is ill-defined (Gilbert & Golberg, 1965). In the present study, the difficulty does not arise as the levels of BHT administration causing SER production are higher than the levels required for effective antioxidant activity in foods, but the practical point is raised of how the dose levels relate to therapeutic and toxic levels of, for example, drugs which produce the SER response. This cannot be more clearly defined until attempts are made to assess the SER response quantitatively and equate it to the enzyme response. Further, Meldolesi (1966) has demonstrated that proliferating SER may be unaccompanied by an enzyme response when the stimulating material is an inhibitor of protein synthesis. It is unlikely, therefore, that if the material under investigation is such an inhibitor, the degree of enzyme response can be directly correlated with toxicity, and in these circumstances SER proliferation would be of dubious toxicological significance. It may be possible, however, to relate an absence of SER proliferation to the low hazard of the drug under investigation if there is no effect on protein synthesis, because if the drug can be metabolized by the enzyme systems already present within the cell, the result can hardly be unphysiological. This argument clearly applies only to those materials having no other toxic effects at the dose levels employed, and is dependent upon the sensitivity of the method employed to detect SER production.

Weibel & Palade (1964) have used magnifications of 61,000 diameters to examine organelles of endothelial cells with a grid containing 525 points, analysing a total of 25,000 points (Weibel *et al.* 1966) for each experimental group. We have analysed 4200 points/experimental group in the first experiment and 10,500/group in the second experiment, but using photomicrographs at 15,000 diameter magnification. At this magnification, SER is readily identified and distinguished from other structures such as convoluted intercellular membranes, with which it may sometimes be confused. The quantitative accuracy depends on the number of points counted and the area of sectioned tissue scanned. We suggest that the method adopted in the second experiment offers a satisfactory compromise, achieving adequate accuracy of analysis with moderate expenditure of time. The use of a computer as described by Weibel *et al.* (1966) allows many more counts to be made in a given time.

It is suggested that the method described offers a satisfactory way of analysing for SER production, and that such analyses are necessary if the toxicological significance of SER proliferation is to be elucidated. Although much of the evidence is in agreement, it cannot yet be stated that SER proliferation is a normal cytological reaction; and even when this is so, the limits of normality will need to be defined. At present a toxic reaction cannot be excluded if SER proliferation results, and it is necessary to equate the degrees of SER production with the response obtained in order to elucidate this relationship.

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## Effets de l'hydroxytoluène butylé sur l'activité enzymatique et l'ultrastructure des hépatocytes du rat

**Résumé**—La réaction de l'aminopyrine-déméthylase, enzyme métabolisant des substances pharmaceutiques, à l'hydroxytoluène butylé (HTB) consommé par des rats pendant 28 jours au plus et à raison de 0–5% de leur ration, a été comparée à la réaction de prolifération du réticulum endoplasmatique lisse (REL) du foie, telle qu'on la mesure par un comptage de points. Les évolutions dans le temps des deux réactions sont comparables mais la réaction enzymatique témoigne d'une plus grande sensibilité. On a d'ailleurs constaté que la réaction enzymatique aux doses les moins élevées ne va pas toujours de pair avec une augmentation soutenue du REL et l'on suggère que celle-ci représente la limite de la réaction physiologique. L'excès de REL disparait rapidement quand on interrompt l'administration de HTB, mais la nature de ce processus n'a pas encore été élucidée. La réincorporation de HTB au régime entraîne une réaction similaire à la réaction initiale.

## Die Wirkung von butyliertem Hydroxytoluol auf die Enzymaktivität und Ultrastruktur von Rattenleberzellen

**Zusammenfassung**—Die Reaktion des Chemikalienstoffwechselenzyms Aminopyrindemethylase auf BHT, das an Ratten bis zu 28 Tage lang in Konzentrationen von 0–5% im Futter verabreicht wurde, ist mit der Proliferationsreaktion des glatten endoplasmatischen Reticulums (SER) in der Leber, bestimmt mittels einer Punktzählmethode, verglichen worden. Der zeitliche Verlauf der Reaktionen ist vergleichbar, aber die Enzymreaktion ist die empfindlichere. Es gibt jedoch Beweise dafür, dass bei den geringeren Dosierungen die Enzymreaktion nicht immer von einer ständigen Vermehrung des SER begleitet zu sein braucht, und es wird die Vermutung ausgesprochen, dass dies die Grenze der physiologischen Reaktion darstellen kann. Auf die Beendigung der BHT-Zufuhr folgt ein rasches Verschwinden des überschüssigen SER durch einen noch unaufgeklärten Prozess. Erneute Verabreichung von BHT im Futter hat eine ähnliche Reaktion wie die ursprüngliche zur Folge.

Food Cosmet. Toxicol. Vol. 3, pp. 387–403. Pergamon Press 1965. Printed in Great Britain

# Absence of Teratogenic Effect of Butylated Hydroxyanisole (BHA) and Butylated Hydroxytoluene (BHT) in Rats and Mice

D. J. CLEGG*

*British Industrial Research Association, Woodmansterne Road, Carshalton, Surrey, England*

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Abstract—Groups of rats and mice of various strains were given butylated hydroxytoluene (BHT; 3,5-di-*tert*-butyl-4-hydroxytoluene) or butylated hydroxyanisole (BHA; 3-*tert*-butyl-4-hydroxyanisole) in accordance with one of 3 different oral dosage regimes. No significant embryotoxic effect was observed, as judged by examination of the skeletal and soft tissues of fully-developed foetuses, as well as by other criteria. The abnormalities encountered were considered to be spontaneous in origin, since they occurred in untreated as well as treated groups. In response to massive doses of vitamin A the experimental animals yielded a wide variety of characteristic abnormalities.

## INTRODUCTION

Brown, Johnson & O'Halloran (1959) reported that in 3 out of 30 litters from rats receiving butylated hydroxytoluene (BHT; 3,5-di-*tert*-butyl-4-hydroxytoluene) at levels up to 0·5% of the diet (approx. 250 mg/kg daily) for 5 months, 10 out of 33 foetuses showed anophthalmia. By sib matings they concluded that the anophthalmia was not due to a genetic factor. No cases of anophthalmia were observed in 600 litters of mice examined.

Brown *et al.* (1959) did not attempt to draw conclusions regarding the possible teratogenic activity of BHT. Nevertheless the inference is clear, and has caused considerable doubt regarding the acceptability of BHT for general use as a food additive in human and animal diets. It was therefore considered that a careful and detailed study was called for, using a variety of dosage regimes in both rats and mice. A similar study was also conducted on the commonly used antioxidant butylated hydroxyanisole (BHA; 3-*tert*-butyl-4-hydroxyanisole).

## EXPERIMENTAL

*Materials.* BHA and BHT were supplied by May and Baker Ltd., Dagenham, Essex and conformed to the same specifications as described by Gilbert & Golberg (1965). Other materials used were commercial samples.

*Experimental design.* Three dosage regimes were used in the experiments: single massive doses on a specific day of gestation, repeated daily doses from the time of mating until sacrifice, and daily doses during the period before mating, continuing through mating and gestation up to the time when the animals were killed. Female animals only were treated. The test materials BHT and BHA were administered as a solution in arachis oil by oral intubation. Control groups comprised animals treated with arachis oil, untreated animals

*Present address: Food and Drug Directorate, Department of National Health and Welfare, Tunney's Pasture, Ottawa 4, Ontario, Canada.

and animals given massive oral doses of vitamin A, the latter serving as a known positive control.

Three strains of albino rat (Tuck, Carworth SPF and Porton SPF), one strain of hooded rat (Benger), and 2 strains of mice (Evans No. 1 and Imperial Chemical Industries (ICI) SPF) were employed in 8 experiments as shown in Table 1.

Table 1. *Summary of animal groupings and dosage*

| Exp. # | Strain | No. of animals* | Test substance | Dose IU | Dose mg/kg | Dose regime |
|---|---|---|---|---|---|---|
| | | | **MICE** | | | |
| 1. | Evans No. 1 | 10 | BHT | | 750 | Daily administration for 32–46 days prior to mating and up to day 12 of pregnancy |
| 2. | Evans No. 1 | 83 | BHT | | 1000 | Single administration on a specific day of pregnancy (day 1–18) |
| 3. | Evans No. 1 | 10 | BHT | | 750 | Daily administration on days 1–18 of pregnancy |
| 4. | ICI SPF | 95 | BHA | | 500 | Daily administration for 7 wk before pairing, continuing until day 18 of pregnancy |
| | | | BHT | | 250, 300, 500 | |
| | | | Vitamin A | 30,000 | | |
| | | | **RATS** | | | |
| 5. | Tuck albino | 32 | BHA | | 750 | Daily administration on days 1–20 of pregnancy† |
| | | | BHT | | 750 | |
| | | | Vitamin A | 5000 | | |
| 6. | Tuck albino<br>Benger hooded | 47 | BHA | | 750 | Daily administration for 70 days before pairing, continuing through pregnancy |
| | | | BHT | | 750 | |
| | | | Vitamin A | 5000 | | |
| 7. | Tuck albino<br>Carworth SPF albino<br>Benger hooded | 111 | BHA | | 1000 | Single administration on day 9, 11 or 13 of pregnancy |
| | | | BHT | | 1000 | |
| | | | Vitamin A | 60,000 | | |
| 8. | Porton SPF albino | 152 | BHA | | 500 | Daily administration for 7 wk before pairing continuing during pregnancy; treatment commenced when rats were 3 or 10 wk old |
| | | | BHT | | 500 | |
| | | | Vitamin A | 30,000 | | |

*Including arachis oil control and untreated control.
†12 of these rats were allowed to continue to parturition, the offspring being examined at weaning (3 wk old).

*Procedure.* In experiments involving the administration of test material during the premating period calculations were made of a Mating Index (no. of matings/no. of opportunities to mate × 100, where one opportunity to mate is considered to be the 5-day oestrous cycle) and a Fertility Index (no. of pregnancies/no. of females mated × 100). Except in the

case of the sixth experiment listed in Table 1, pregnancies were all terminated prior to parturition. In mice, day 1 of pregnancy was determined by the presence of a vaginal plug, and pregnancy was then terminated on day 18. In rats, day 1 was determined by the presence of semen in the vaginal smear and pregnancy was terminated on day 20. At termination of pregnancy, both ovaries and uterus were removed from the anaesthetized rat. The number of corpora lutea of pregnancy in each ovary was noted. The numbers and positions of implantation sites (including resorption sites) in the separate uterine horns were recorded. The foetuses were removed, examined for gross abnormalities, sexed (by measuring the anogenital distance), weighed, and fixed for 4 days in 70% alcohol (after evisceration) or in Bouin's fluid.

From each rat uterus, alternate foetuses, fixed in 70% alcohol, were stained with alizarin, for examination of skeletal abnormalities; the others, fixed in Bouin, were transferred to 70% alcohol before being cut into 15 standard slices, according to the technique of Wilson (1965) for examination of soft tissue abnormalities. The majority of mouse foetuses were stained with alizarin.

## RESULTS

The results of experiments employing Evans No. 1 mice are presented in Tables 2 and 3. The only malformation observed was microphthalmia. No soft tissue examination was performed on any of the foetuses.

When ICI SPF mice were used (Table 4), it was initially intended to administer 500 mg/kg of BHA or BHT. However, an unduly high mortality (85% after 2-wk treatment) resulted with BHT (cf. 5% mortality with BHA). The dose of BHT was therefore reduced to 300 mg/kg, but had to be further reduced when the mortality was still found to be 70%. The final dose used was 250 mg/kg, which gave a 33% mortality after 7-wk treatment. (cf. 25% mortality with 500 mg BHA/kg after 7 wk). The body weight of surviving animals was well maintained during the period before mating.

The number of abortions amongst the ICI SPF mice was unexpectedly high. In mice aborting after treatment with BHA there were 74 implantation sites, but only 40 corpora lutea of pregnancy were observed. Twenty-three aborted embryos were examined and showed no abnormality; 9 of these had open eyes, in keeping with the stage of development. In the 3 survivors of the group given 500 mg BHT/kg, the single aborting animal showed 13 implantation sites, and 12 corpora lutea of pregnancy; of the 5 survivors at 300 mg BHT/kg one mouse showed 12 implantation sites and 10 corpora lutea of pregnancy. All embryos examined were normal. At the lowest dose of BHT (250 mg/kg) the 2 aborting mice showed 23 implantation sites and 25 corpora lutea of pregnancy; 13/23 embryos examined had open eyes. In the untreated control group the aborting mice (2/9) showed 24 implantation sites, and 19 corpora lutea of pregnancy; 5/24 aborted embryos examined had open eyes.

The results of tests with Tuck and Carworth SPF albino rats given single doses on specific days of pregnancy are shown in Table 5. After vitamin A treatment, the abnormalities in Tuck rats included exencephaly, micromelia, anophthalmia, cleft palate and open eyes. The incidence of abnormalities in Carworth SPF rats is set out in detail in Table 6.

Overall conception rates in Benger hooded rats were low as a result of uterine infection. Hence only 2 animals treated with BHT mated successfully; all the foetuses were found to be normal.

Two groups of albino rats (Tuck) were treated with one or other of the test substances

Table 2. *Effect of 750 mg BHT/kg administered daily to Evans No. 1 mice*

| Test substance | Duration of treatment (days) | No. surviving in group | Mating Index (%) | Fertility Index (%) | No. aborted | No. of corpora lutea | No. of foetuses | | | | Average weight of foetuses (g) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Male | Female | Resorbed | Abnormal | Male | Female | Group |
| **Administration before and during pregnancy** | | | | | | | | | | | | | |
| BHT | 50–64 | 7/7 | 88 | 29 | 0 | 28 | 14 | 11 | 0 | 0 | 1·28 | 1·19 | 1·24 |
| Arachis oil (control) | 63 | 3/3 | 75 | 33 | 0 | 11 | 6 | 2 | 8 | 0 | 1·35 | 1·25 | 1·31 |
| **Administration during pregnancy** | | | | | | | | | | | | | |
| BHT | 17 | 4/5 | — | 25 | 0 | 15 | 1 | 12 | 3 | 0 | 0·89 | 0·88 | 0·88 |
| Arachis oil (control) | 17 | 4/5 | — | 75 | 0 | 44 | 19 | 13 | 2 | 0 | 1·03 | 1·10 | 1·06 |

[illegible] from the onset of pregnancy. One group was killed on day 20 of pregnancy and the second group was treated until parturition, the young being examined at 3 wk of age. Post-natal teratogenic effects similar to those described by Paget & Thorpe (1964) for sulphonamides were not encountered. The results are given in Tables 7 and 8.

In 4 groups of rats, administration of the test compounds commenced before mating and continued until pregnancy was terminated on day 20. Two groups of albino rats (Tuck and Sprague hooded) were treated for 10 wk before pairing. The results are shown in Tables 9 and 10. Weight loss was observed in albino rats treated with BHA. The remaining 2 groups were Porton SPF albino rats treated for 7 wk before pairing, one group commencing treatment at weaning (3 wk old) and the second when 10 wk old. The results are shown in Tables 11, 12 and 13. Growth was impaired in rats given BHA or vitamin A.

Table 3. *Effect of a single dose of 1000 mg BHT/kg at different stages of pregnancy of Evans No. 1 mice*

| Test substance | Day of treatment | No. in group | No. pregnant | No. of corpora lutea | No. of foetuses* | | | Average weight of foetuses (g) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Male | Female | Resorbed | Male | Female | Group |
| BHT | 18 | 2 | 2 | 31 | 21 | 5 | 0 | 1·25 | 1·22 | 1·23 |
| Arachis oil | 18 | 2 | 2 | 29 | 13 | 11 | 4 | 1·29 | 1·31 | 1·30 |
| BHT | 17 | 2 | 2 | 27 | 10 | 9 | 3 | 1·03 | 1·32 | 1·18 |
| Arachis oil | 17 | 2 | 1 | 10 | 6 | 3 | 1 | 1·01 | 0·94 | 0·99 |
| BHT | 16 | 2 | 1 | 15 | 5 | 7 | 0 | 0·92 | 0·99 | 0·96 |
| Arachis oil | 16 | 2 | 1 | 20 | 9 | 6 | 0 | 1·07 | 0.99 | 1·03 |
| BHT | 15 | 2 | 1 | 12 | 6 | 6 | 0 | 1·36 | 1·35 | 1·36 |
| Arachis oil | 15 | 2 | 1 | 12 | 10 | 2 | 0 | 1·44 | 1·45 | 1·44 |
| Arachis oil | 14† | 2 | 1 | 11 | 4 | 3 | 0 | 0·89 | 0·88 | 0·89 |
| BHT | 13 | 3 | 2 | 33 | 9 | 13 | 4 | 0·96 | 0·98 | 0·98 |
| Arachis oil | 13 | 2 | 1 | 15 | 4 | 6 | 1 | 1·29 | 1·20 | 1·22 |
| BHT | 12 | 2 | 2 | 30 | 11 | 13 | 3 | 1·04 | 0·99 | 1·02 |
| Arachis oil | 12 | 2 | 1 | 13 | 6 | 6 | 0 | 1·08 | 1·10 | 1·09 |
| Arachis oil | 11† | 2 | 1 | 16 | 7 | 6 | 0 | 1·01 | 0·98 | 0·99 |
| BHT | 10 | 3 | 3 | 43 | 26 | 12 | 1 | 1·00 | 1·01 | 1·00 |
| Arachis oil | 10 | 3 | 2 | 27 | 16 | 8 | 1 | 1·08 | 1·03 | 1·06 |
| BHT | 9 | 3 | 2 | 27 | 13 | 9 | 3 | 0·68 | 0·68 | 0·68 |
| Arachis oil‡ | 9 | 2 | 1 | 11 | – | – | – | – | – | – |
| BHT | 8 | 3 | 2 | 34 | 11 | 16 | 4 | 1·19 | 1·14 | 1·16 |
| Arachis oil | 8 | 2 | 2 | 25 | 9 | 9 | 2 | 1·24 | 1·24 | 1·24 |
| BHT | 6§ | 3 | 2 | 31 | 11 | 16 | 0 | 1·06 | 1·07 | 1·07 |
| Arachis oil | 6 | 2 | 2 | 25 | 14 | 9 | 2 | 1·25 | 1·17 | 1·22 |
| BHT | 5 | 2 | 1 | 13 | 6 | 4 | 2 | 0·98 | 0·99 | 0·98 |
| Arachis oil | 5 | 2 | 1 | 13 | 7 | 5 | 0 | 1·05 | 1·00 | 1·03 |
| BHT | 4‖ | 3 | 1 | 13 | 5 | 5 | 0 | 0·93 | 0·92 | 0·93 |
| BHT | 3 | 2 | 2 | 25 | 12 | 10 | 2 | 0·94 | 0·94 | 0·94 |
| Arachis oil | 3 | 2 | 1 | 16 | 7 | 8 | 2 | 0·99 | 0·99 | 0·99 |
| BHT | 2 | 3 | 2 | 26 | 18 | 8 | 2 | 1·07 | 0·92 | 1·02 |
| Arachis oil | 2 | 2 | 1 | 13 | 3 | 7 | 1 | 1·16 | 1·21 | 1·19 |
| BHT | 1 | 3 | 1 | 14 | 5 | 5 | 3 | 1·46 | 1·27 | 1·36 |
| Arachis oil | 1 | 2 | 2 | 27 | 10 | 14 | 0 | 1·28 | 1·41 | 1·35 |
| None | – | 2 | 2 | 23 | 7 | 9 | 0 | 1·21 | 1·15 | 1·18 |

*There was only 1 abnormal foetus which occurred in the group given BHT on day 3 of pregnancy. This foetus showed microphthalmia.
†Animals treated with BHT on this day failed to conceive.
‡There was only 1 abortion which occurred in arachis oil group on day 9 of treatment.
§On day 7, all animals were found not to have conceived.
‖Animals given arachis oil on this day failed to conceive.

Table 4. *Effect of BHA, BHT, vitamin A or arachis oil administered daily, to ICI SPF mice before and during pregnancy*

| Test substance | Dose | Duration of treatment (days) | No. surviving in group | Mating Index (%) | Fertility Index (%) | No. aborted* | No. of corpora lutea | No. of foetuses | | | | Average weight of foetuses (g) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Male | Female | Resorbed | Abnormal | Male | Female | Group |
| BHA | 500 mg/kg | 80–94 | 15/20 | 55 | 75 | 7 | 27 | 6 | 13 | 2 | 1 dead | 1·27 | 1·29 | 1·28 |
| BHT | 500 mg/kg | 82–120 | 3/20 | 30 | 100 | 1 | 22 | 3 | 6 | 6 | 1† | 1·34 | 1·50 | 1·45 |
| BHT | 300 mg/kg | 83–95 | 5/20 | 100 | 60 | 1 | 28 | 16 | 10 | 0 | 0 | 1·34 | 1·25 | 1·30 |
| BHT | 250 mg/kg | 78–87 | 10/15 | 100 | 90 | 2 | 103 | 31 | 34 | 16 | 0 | 1·37 | 1·34 | 1·36 |
| Vitamin A | 30,000 IU | 68–71 | 6/10 | 84 | 0 | – | – | – | – | – | – | – | – | – |
| Arachis oil | 5 ml/kg | 84–92 | 10/10 | 84 | 100 | 0 | 120 | 50 | 50 | 9 | 2‡ | 1·44 | 1·37 | 1·40 |
| None | — | — | 9/9 | 60 | 100 | 2 | 111 | 31 | 23 | 14 | 0 | 1·42 | 1·24 | 1·35 |

*Details of number of corpora lutea, implantations and abnormalities of aborted individuals are given in the text.

†1 foetus with no 5th sternal ossification centre.

‡1 foetus with diaphragmatic hernia, and one with no 5th sternal ossification centre.

Table 5. Effect of single doses of 1000 mg/kg of BHA, or BHT, vitamin A (50,000 IU) or arachis oil administered to albino rats on a specific day of pregnancy

| Test substance | Day of treatment | Fertility Index (%) | No. of corpora lutea | No. of foetuses | | | | No. of runts | Average weight of foetuses | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Male | Female | Resorbed | Abnormal | | Male | Female | Group |
| **Tuck: 1 rat/treatment** | | | | | | | | | | | |
| BHA | 9 | | 9 | 5 | 3 | 0 | 0 | 0 | 1·99 | 2·06 | 2·02 |
| BHT | 9 | | 15 | 6 | 5 | 0 | 0 | 0 | 2·19 | 2·09 | 2·14 |
| Vitamin A | 9 | | 14 | 4 | 8 | 1 | 10 | 0 | 2·01 | 2·09 | 2·05 |
| BHA | 11 | | 1 | 0 | 0 | 1 | 0 | 0 | — | — | — |
| BHT | 11 | | 12 | 7 | 3 | 2 | 0 | 0 | 2·28 | 2·29 | 2·29 |
| Vitamin A | 11 | | 11 | 6 | 4 | 0 | 3 | 0 | 2·29 | 2·24 | 2·27 |
| BHA | 13 | | 8 | 4 | 4 | 0 | 0 | 0 | 3·83 | 3·99 | 3·91 |
| BHT | 13 | | 15 | 10 | 3 | 0 | 0 | 0 | 2·25 | 2·32 | 2·29 |
| Vitamin A | 13 | | 10 | 3 | 2 | 1 | 1 | 0 | 3·71 | 3·34 | 3·53 |
| **Carworth SPF: 6 rats/treatment** | | | | | | | | | | | |
| BHA | 9 | 83 | 73 | 33 | 30 | 0 | 4 | 1 | 4·5 | 4·2 | 4·4 |
| BHT | 9 | 100 | 93 | 42 | 32 | 0 | 4 | 0 | 4·4 | 4·1 | 4·3 |
| Vitamin A | 9 | 100 | 93 | 36 | 34 | 3 | 37 | 4 | 4·2 | 3·9 | 4·1 |
| Arachis oil | 9 | 100 | 82 | 42 | 35 | 0 | 5 | 5 | 4·4 | 4·3 | 4·4 |
| BHA | 11 | 100 | 89 | 41 | 34 | 2 | 5 | 0 | 4·5 | 4·5 | 4·5 |
| BHT | 11 | 100 | 87 | 38 | 35 | 7 | 1 | 0 | 4·3 | 4·0 | 4·1 |
| Vitamin A | 11 | 100 | 93 | 33 | 33 | 5 | 18† | 2 | 4·3 | 4·2 | 4·3 |
| Arachis oil | 11 | 83 | 71 | 33 | 28 | 4 | 5 | 0 | 4·6 | 4·3 | 4·5 |
| BHA | 13 | 100 | 94 | 42 | 37 | 4 | 9 | 1 | 4·3 | 4·2 | 4·3 |
| BHT | 13 | 100 | 83 | 31 | 42 | 4 | 2 | 0 | 4·4 | 4·2 | 4·3 |
| Vitamin A | 13 | 100 | 84 | 41 | 29 | 3 | 14 | 3 | 4·3 | 4·1 | 4·2 |
| Arachis oil | 13 | 67 | 60 | 26 | 27 | 1 | 0 | 2 | 4·3 | 4·0 | 4·2 |
| None | — | 86 | 169 | 69 | 79 | 7 | 1 | 1 | 4·3 | 4·2 | 4·3 |

*In Tuck albino rats the abnormalities induced by vitamin A included exencephaly, micromelia, anophthalmia, open eyes and cleft palate. The incidence and types of abnormalities induced in Carworth SPF rats are shown in Table 6.

†In addition 7 other abnormal embryos aborted.

Table 6. *Incidence and types of foetal abnormalities in Carworth SPF rats given single doses* of BHA, BHT, vitamin A or arachis oil on a specific day of pregnancy*

| Test substance | Day of treatment | Proportion of rats with abnormal foetuses | Total no. of abnormal foetuses | Type of abnormality: Gross examination | | | Skeletal examination | | | Sternal ossification centres | | | Soft tissue examination | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Exencephalus | Absence of tail | Anophthalmia | Elongated mandible | 14 ribs | Vertebral fusion | Absence of fifth | Absence of fifth and sixth | Supernumerary centres | Hydronephrosis | Hydrocephalus | Microphthalmia | Duplicated left carotid | Absence of iliac artery | Agenesis of testes | Reduced auricles | Liver enlargement |
| BHA | 9 | 3/6 | 4 | | | | 3 | | | | | | 1 | | | | | | | |
| BHT | 9 | 2/6 | 4 | | | | 2 | | | 2 | | | | | | | | | | |
| Vitamin A | 9 | 6/6 | 37 | 21 | | 2 | 8 | | 1 | 1 | | | 4 | 9 | 1 | 1 | | 1 | | |
| Arachis oil | 9 | 3/6 | 5 | | | | 4 | | | 1 | | | | | | | | | | |
| BHA | 11 | 3/6 | 5 | | | | 3 | | | | 1 | | 1 | | | | | | | |
| BHT | 11 | 1/6 | 1 | | | | | | | | | | | | | | 1 | | | |
| Vitamin A | 11 | 6/6 | 18 | 7 | | | 11 | 1 | | | | | | | | | | | | |
| Arachis oil | 11 | 3/6 | 5 | | 1 | | 2 | 1 | | 1 | | | | | | | 1 | 1 | 1 | 1 |
| BHA | 13 | 3/6 | 9 | | | | 6 | | | 2 | | 1 | | | | | | | | |
| BHT | 13 | 1/6 | 2 | | | | 2 | | | | | | | | | | | | | |
| Vitamin A | 13 | 6/6 | 14 | | | | 12 | | | 1 | | | 3 | | | | | | | |
| Arachis oil | 13 | 0/6 | 0 | | | | | | | | | | | | | | | | | |
| None | — | 1/14 | 1 | | | | | | | | | | 1 | | | | | | | |

*Doses are as for Table 5.
Blank spaces denote negative findings.

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**Table 7.** *Effect* of repeated daily doses of 750 mg/kg of BHA or BHT, vitamin A (5000 IU) or arachis oil administered to Tuck albino rats during pregnancy*

| Test substance | No. surviving in group | No. of corpora lutea | No. of foetuses† | | | Average weight of foetuses (g) | | |
|---|---|---|---|---|---|---|---|---|
| | | | Male | Female | Resorbed | Male | Female | Group |
| BHA | 3/5‡ | 33 | 13 | 10 | 1 | 2·97 | 2·77 | 2·89 |
| BHT | 5/5 | 55 | 25 | 21 | 1 | 3·49 | 3·45 | 3·47 |
| Vitamin A | 5/5 | 51 | 28 | 20 | 1 | 3·48 | 3·48 | 3·48 |
| Arachis oil | 5/5 | 50 | 21 | 15 | 0 | 3·67 | 3·30 | 3·51 |

*There were no abortions and the Fertility Index was 100% in all groups.

†There were no abnormal foetuses.

‡Of the 2 rats that died (one on day 3 and one on day 11) the first showed corpora lutea of pregnancy and the second 11 apparently normal embryos.

**Table 8.** *Results of examination of 3-wk-old Tuck albino rats after daily maternal treatment throughout pregnancy with 750 mg/kg of BHA or BHT, vitamin A (5000 IU) or arachis oil*

| Test substance | Maternal rats | | No. of offspring | | |
|---|---|---|---|---|---|
| | No. treated | No. pregnant | At parturition | At 3 wk | Abnormal |
| BHA | 3 | 2 | 19 | 13 | 0 |
| BHT | 3 | 2 | 16 | 12 | 0 |
| Vitamin A | 3 | 2 | 22 | 10 | 2* |
| Arachis oil | 3 | 2 | 17 | 14 | 0 |

*Non-patent anus.

Table 9. *Effect of 750 mg/kg of BHA or BHT, vitamin A (5000 IU) or arachis oil administered daily to rats for 10 wk before and during pregnancy*

| Test substance | No. surviving in group | Mating Index (%) | Fertility Index (%) | No. aborted | No. of corpora lutea | No. of foetuses | | | | Average weight of foetuses (g) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Male | Female | Resorbed | Abnormal | Male | Female | Group |
| **Tuck albino rats** | | | | | | | | | | | | |
| BHA | 1/4 | 100 | 100 | 0 | 10 | 3 | 4 | 1 | 0 | 2·39 | 2·09 | 2·18 |
| BHT | 8/8 | 89 | 57 | 0 | 43 | 16 | 21 | 4 | 0 | 2·18* | 1·92* | 2·00 |
| Vitamin A | 7/8 | 70 | 86 | 0 | 63 | 21 | 29 | 0 | 0 | 2·34† | 2·26† | 2·29 |
| Arachis oil | 4/4 | 80 | 75 | 0 | 33 | 10 | 18 | 3 | 1‡ | 1·99 | 2·13 | 2·09 |
| None | 6/6 | 100 | 83 | 0 | 59 | 23 | 27 | 2 | 1§ | 2·31 | 2·20 | 2·27 |
| **Benger hooded rats** | | | | | | | | | | | | |
| BHA | 0/3 | – | – | – | – | – | – | – | – | – | – | – |
| BHT | 3/5 | 5 | 33 | 0 | 14 | 1 | 2 | 2 | 0 | 1·68 | 1·68 | 1·68 |
| Vitamin A | 4/5 | 9 | 25 | 0 | 1‖ | 0 | 0 | 5 | – | – | – | – |
| Arachis oil | 2/2 | 100 | 100 | 0 | 34 | 0 | 0 | 19 | – | – | – | – |
| None | 2/2 | 100 | 0 | – | – | – | – | – | – | – | – | – |

*Two animals littered spontaneously; weights of offspring not included in Table.
†One animal littered spontaneously; weights of offspring not included in Table.
‡Hydronephrosis.
§Duplicated left carotid arteries.
‖Impossible to count because of pathological changes.

Table 10. *Group mean body weights of rats treated for 10 wk before pairing*

| Test substance | Dose | No. of rats | Initial body weight (g) | Mean body weight (g) at end of wk | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| | | | **Tuck albino rats** | | | | | | | | | | |
| BHA | 750 mg/kg | 4 | 208 | 188 | 198 | 195 | 198 | 183 | 179 | 180 | 165 | 168 | 183 |
| BHT | 750 mg/kg | 8 | 221 | 220 | 224 | 221 | 226 | 223 | 213 | 216 | 223 | 228 | 226 |
| Vitamin A | 5000 IU | 8 | 218 | 223 | 229 | 229 | 230 | 231 | 228 | 235 | 233 | 240 | 239 |
| Arachis oil | 2 ml/kg | 4 | 240 | 227 | 235 | 235 | 235 | 240 | 235 | 241 | 235 | 243 | 243 |
| | | | **Benger hooded rats** | | | | | | | | | | |
| BHA | 750 mg/kg | 3 | 280 | 257 | 257 | 257 | 245* | 230 | 260* | 250 | 260 | —* | — |
| BHT | 750 mg/kg | 5 | 268 | 260 | 268 | 262 | 262 | 254 | 260 | 260 | 264 | 273* | 273 |
| Vitamin A | 5000 IU | 5 | 278 | 280 | 280 | 282 | 288 | 290 | 283 | 281 | 278 | 303* | 295 |
| Arachis oil | 2 ml/kg | 2 | 275 | 275 | 280 | 280 | 285 | 285 | 275 | 280 | 285 | 290 | 290 |

*1 rat died.

Table 11. *Effect of daily doses of 500 mg/kg of BHA or BHT, vitamin A (30,000 IU) or arachis oil administered to Porton SPF rats for 7 wk before and during pregnancy*

| Test substance | No. surviving in group | Mating Index (%) | Fertility Index (%) | No. aborted | No. of corpora lutea | No. of foetuses | | | | No. of runts | Average weight of foetuses (g) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Male | Female | Resorbed | Abnormal | | Male | Female | Group |
| **Commenced treatment at weaning (3 wk)** | | | | | | | | | | | | | |
| BHA | 20/20 | 83 | 100 | 0 | 216 | 84 | 96 | 22 | 21 | 5 | 3·8 | 3·5 | 3·6 |
| BHT | 18/20 | 72 | 100 | 0 | 206 | 72 | 78 | 7 | 11 (+5 dead) | 6 | 3·6 | 3·3 | 3·5 |
| Vitamin A | 19/20 | 74 | 90 | 3 | 131 | 42 | 64 | 6 | 74 | 8 | 3·6 | 3·5 | 3·5 |
| Arachis oil | 20/20 | 77 | 100 | 1 | 228 | 96 | 94 | 14 | 11 | 3 | 3·8 | 3·5 | 3·6 |
| None | 19/20 | 70 | 84 | 0 | 191 | 78 | 84 | 8 | 28 | 5 | 3·3 | 3·3 | 3·3 |
| **Commenced treatment at 10 wk** | | | | | | | | | | | | | |
| BHA | 11/12 | 100 | 100 | 0 | 131 | 40 | 62 | 15 | 10 | 3 | 3·9 | 3·7 | 3·8 |
| BHT | 7/10 | 88 | 71 | 0 | 50 | 20 | 22 | 4 | 2 | 0 | 3·4 | 3·3 | 3·4 |
| Vitamin A* | 8/10 | 67 | 100 | 2 | 44 | 15 | 18 | 3 | 27 | 0 | 3·8 | 4·1 | 4·0 |
| Arachis oil | 10/10 | 90 | 100 | 0 | 101 | 37 | 38 | 3 | 3 | 2 | 3·6 | 3·4 | 3·5 |
| None | 10/10 | 100 | 100 | 0 | 119 | 46 | 55 | 2 | 6 | 0 | 3·9 | 3·6 | 3·7 |

*1 rat died prematurely, and has been omitted from the above Table; 7 abnormal embryos were found at autopsy. These abnormalities are detailed in Table 13.

Table 12. *Group mean body weights of Porton rats treated for 7 wk before pairing*

| Test substance | No. in group | Dose | Body weight (g) at end of wk | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| | | **Commenced treatment at weaning (3 wk)** | | | | | | | | |
| BHA | 20 | 500 mg/kg | 66 | 94 | 121 | 144 | 160 | 168 | 178 | 185* |
| BHT | 20 | 500 mg/kg | 63 | 85 | 118 | 145 | 160 | 180 | 192 | 203 |
| Vitamin A | 20 | 30,000 IU | 67 | 96 | 126 | 146 | 167 | 177 | 183 | 184 |
| Arachis oil | 20 | 2 ml/kg | 68 | 98 | 129 | 152 | 177 | 189 | 202 | 215 |
| None | 20 | — | 72 | 103 | 131 | 151 | 170 | 186 | 204 | 211 |
| | | **Commenced treatment at 10 wk** | | | | | | | | |
| BHA | 12 | 500 mg/kg | 182 | 185 | 192 | 200 | 210 | 213 | 217 | 221 |
| BHT | 10 | 500 mg/kg | 198 | 192† | 196 | 198 | 210 | 212 | 226 | 226 |
| Vitamin A | 10 | 30,000 IU | 181 | 190 | 190 | 195 | 204 | 201 | 203 | 205 |
| Arachis oil | 10 | 2 ml/kg | 185 | 196 | 198 | 203 | 211 | 214 | 220 | 223 |
| None | 10 | — | 186 | 200 | 206 | 212 | 218 | 224 | 230 | 233 |

*1 rat died during the preceding wk.
†3 rats died during the preceding wk.

Table 13. *Incidence and types of foetal abnormalities in Porton SPF albino rats given repeated daily doses* of BHA, BHT, vitamin A or arachis oil*

| Test substance | Proportion of rats with abnormal foetuses | Total no. of abnormal foetuses | Type of abnormality: Gross examination | | | Skeletal examination | | | | | | | Sternal ossification centres | | | | | Soft tissue examination | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Exencephalus | Anophthalmia | Open eyes | Elongated mandible | 12 ribs | 14 ribs | Absence of tooth buds | Absence of ischium | Agenesis of hind limbs | Spina bifida | Absence of fifth | Absence of sixth | Absence of fifth and sixth | Supernumerary centres | No ossification centres | Hydronephrosis | Hydrocephalus | Meningeal haemorrhage | Microphthalmia | Macrophthalmia | Cleft palate | Diaphragmatic hernia | Situs inversus stomach | Liver agenesis |
| **Daily doses from 3 wk of age for 7 wk before and during pregnancy** | | | | | | | | | | | | | | | | | | | | | | | | | | |
| BHA | 10/20 | 21 | | | | 9 | 1 | 1 | | | | | 4 | 1 | 4 | 1 | | | | | | | | | | |
| BHT | 11/18 | 11 | | | | 1 | 1 | | | 1 | | | | | 8 | | 1 | | | | | | | | | |
| Vitamin A | 13/15 | 74 | 18 | 6 | 20 | 44 | | 1 | 1 | | | | | | 5 | | | 2 | | 1 | 1 | 7 | 30 | 1 | | |
| Arachis oil | 6/19 | 11 | | | | | | | | | | | 2 | | 8 | 1 | | | | | | | | | | |
| None | 11/17 | 28 | | | | 2 | 1 | 1 | | | | | 1 | 6 | 15 | 1 | 1 | | | | | | | | 1 | |
| **Daily doses from 10 wk of age for 7 wk before and during pregnancy** | | | | | | | | | | | | | | | | | | | | | | | | | | |
| BHA | 6/11 | 10 | | | | 3 | | | | | | | 5 | | 2 | | | | | | | | | | | |
| BHT | 2/7 | 2 | | | | | | | | | | | 1 | | 1 | | | | | | | | | | | |
| Vitamin A | 6/6 | 34 | 13 | 11 | 2 | 6 | 1 | | 1 | | 2 | 1 | | | 4 | | | 2 | 1 | | 1 | 1 | 15 | 1 | | 1 |
| Arachis oil | 3/10 | 3 | | | | 1 | | | | | | | | | 1 | | | | | | | | | | 1 | |
| None | 4/10 | 6 | | | | 2 | | | | | | | 4 | | | | | | | | | | | | | |

*Doses are as for Table 11.
Blank spaces denote negative findings.

A check on possible effects on resorption rate (Table 14) revealed no significant trend on the part of any treatment in agreement with the findings of Telford, Woodruff & Linford (1952). The high incidence of resorptions in ICI SPF mice is probably associated with the unexplained regression observed in the corpora lutea of these animals.

Table 14. *Summary of overall incidence of resorptions in different strains of rats and mice*

| Species | Strain | Resorptions, as percentage of total litter | | | | |
|---|---|---|---|---|---|---|
| | | Untreated | Arachis oil | BHA | BHT | Vitamin A |
| Mice | Evans No. 1 | 8·2 | — | — | 8·0 | — |
| | ICI SPF | 20·1 | 9·0 | 9·8 | 22·0 | — |
| Rats | Tuck | 2·0 | 4·5 | 6·3 | 5·7 | 1·0 |
| | Carworth SPF | 4·5 | 2·6 | 2·7 | 4·8 | 5·1 |
| | Porton SPF | 3·7 | 6·0 | 11·6 | 5·4 | 6·1 |

## DISCUSSION

*Findings in rats*

The results presented above fail to confirm 2 of the findings reported in hooded rats by Brown *et al.* (1959). These authors administered BHT in the diet at a level of 0·5% for 5 months and reported depilation in adult rats as well as the occurrence of foetal anophthalmia. In the present experiments, in which rats were given daily doses of BHT for an average of 85 days (max 120 days) depilation was not seen and foetal abnormalities were limited to one instance of delayed skeletal development.

It seems possible that the results obtained by Brown *et al.* (1959) are attributable to an unsuspected dietary deficiency or imbalance. It may be considered, for example, whether there was a lack of vitamin A or E. It is estimated that the diet provided 8800 IU vitamin A/kg (calculated from Moore, 1957) and 39 mg vitamin E/kg (calculated from McCance & Widdowson, 1960). Assuming a dietary intake of 15 g/rat/day, the vitamin A level is adequate (132 IU/day). The dietary content of vitamin E, however, is only 0·59 mg/day. Spector (1956) quotes a vitamin E requirement in unstressed rats of 1·5 mg/rat/day, whilst Albritton (1954) states that the requirement during growth is 3 mg/rat/day. J. Green (personal communication in 1965), however, feels that these figures are too high, due possibly to oxidative destruction of vitamin E in the diet, which was not considdere by earlier workers; or perhaps due to the early pre-occupation with the pregnancy test. A stock breeding diet containing 15% wheat germ and 5% of arachis oil (the only added fat) was found by assay to have a content of only 10 $\mu$g/g of vitamin E (J. Green, personal communication in 1965). Moore & Sharman (1961) showed that rats were fertile in the pregnancy test on a diet containing 10% of lard, even when only 22 mg/kg of vitamin E was added. It would seem unlikely that a diet containing 38 mg/kg of vitamin E, even with 20% lard present, would produce signs of deficiency of the type described by Brown *et al.* (1959). It is true that the rolled oats and crushed wheat would contribute additional, and highly unsaturated fat to some degree, but this fact must be balanced against the presence of adequate antioxidants in the diet to prevent destruction in the gut.

Furthermore, the manifestations do not appear to fit vitamin E deficiency in the rat. There was no evidence of sterility; in the experience of most workers, failure of reproduction

is diagnostic. Other signs of vitamin E deficiency (with the possible exception of creatinuria) are unlikely to be present in rats which still have enough vitamin E to produce live young. No published reference has been found to the loss of hair in vitamin E deficiency. Several workers have described the involvement of vitamin E deficiency in the causation of ocular lesions; Green (1964) suggests that lack of vitamin A may be a primary cause in several of the cases. Anophthalmia has not been described, as such, in vitamin E deficiency. Thomas & Cheng (1952) and Cheng & Thomas (1953) have provided a detailed account of the congenital abnormalities produced in rats on marginally-inadequate amounts of vitamin E. These included cleft palate, facial fissures, umbilical hernia, hydrocephalus, exencephaly and other disturbances of the skeletal system. Ocular lesions were not described.

Avitaminosis A (Warkany & Schraffenberger, 1944) could account for the anophthalmia reported by Brown *et al.* (1959). Equally, avitaminosis A affects hair growth in rats (Gudjonsson, 1930) and although the histological description of the affected skin in the report by Brown *et al.* (1959) is by no means consistent with the typical picture (Studer & Frey, 1949), it is of interest to note that, according to the results of Brown *et al.* (1959) the depilation seemed to accompany times of natural stress (growth, reproduction) and cleared up sometime after termination of the stress. There is thus a good correlation with the vitamin A requirements at such times.

A vitamin A intake of 132 IU/day is a good excess over requirement and is unlikely to be substantially destroyed in the gut. Similarly, extensive destruction is unlikely to have occurred in the prepared diet (Johnson, O'Halloran & Hewgill, 1958). A tendency for reduction of liver weight, with maintenance of a constant ratio of liver weight to body weight, has been noted in vitamin A deficiency (Morton & Phillips, 1959; Diplock, Green & Bunyan, 1965). In the experiments of Brown *et al.* (1959) the hepatomegaly induced by the administration of BHT would mask any such effect. Unfortunately these authors did not report the changes in the absolute and relative liver weight of untreated control animals during the course of the experiments.

*Findings in mice*

In the case of mice, Brown *et al.* (1959) observed none of the effects described for rats. Considering the present experiments on Evans No. 1 mice, one case of microphthalmia was encountered following administration of 1000 mg BHT/kg on day 3 of pregnancy. At this stage in mouse development, implantation has not taken place, the neural plate is not present and since the eyes develop as outpushings of the brain, it seems improbable that the abnormality was caused by BHT; similar doses failed to have any effect especially on days 9, 10 and 11 when the eyes are developing and thus most likely to be susceptible. This abnormality is therefore considered to have occurred spontaneously.

## CONCLUSION

Under the conditions of these experiments neither BHA nor BHT showed evidence of embryotoxicity. BHT was more toxic to female mice than BHA. On the other hand BHA retarded the growth of weanling albino female rats and caused weight loss in adults, whereas BHT, at the same high level of dosage, had no such effect.

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## Absence d'effet tératogène de l'hydroxyanisol butylé (BHA) et de l'hydroxytoluène butylé (BHT) sur les rats et les souris


**Résumé**—On a soumis des lots de rats et souris de races diverses, à 3 doses orales différente de l'hydroxytoluène butylé (BHT; 3,5-di-*tert*-butyl-4-hydroxytoluène) ou d'hydroxyanisol butylé (BHA; 3-*tert*-butyl-4-hydroxyanisol). On n'a pas observé d'effets embryotoxiques importants, à en juger par l'examen des tissus osseux et des tissus mous de foetus complètement développés, ainsi que par d'autres critères. On a considéré que les anomalies constatées avaient une origine spontanée, puisqu'on les observait aussi bien dans des groupes d'animaux traites que dans des groupes non traités. En réponse à des doses massives de vitamines A, les animaux expérimentaux présentaient des anomalies caractéristiques variées.


## Das Fehlen der teratogenen Wirkung von butyliertem Hydroxyanisol (BHA) und butyliertem Hydroxytoluol (BHT) bei Ratten und Mäusen


**Zusammenfassung**—Gruppen von Ratten und Mäusen verschiedener Stämme erhielten nach einem von 3 verschiedenen oralen Dosierungsschemata butyliertes Hydroxytoluol (BHT; 3,5-Di-*tert*-butyl-4-hydroxytoluol) oder butyliertes Hydroxyanisol (BHA; 3-*Tert*-butyl-4-hydroxyanisol). Es wurden keine signifikanten embryotoxischen Wirkungen bei der Untersuchung des Skeletts und der Weichteile vollentwickelter Föten und in bezug auf andere Kriterien beobachtet. Die festgestellten Anomalien wurden für spontan gehalten, da sie ebenso bei behandelten wie bei unbehandelten Gruppen auftraten. Infolge massiver Dosen von Vitamin A traten bei den Versuchstieren die unterschiedlichsten charakteristischen Anomalien

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## SHORT PAPER

# The BHT Content of Human Adipose Tissue

A. J. COLLINGS* and M. SHARRATT†

*Department of Medical Biochemistry and Pharmacology, University of Birmingham, Edgbaston, Birmingham 15, England*

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**Summary**—A method for determining butylated hydroxytoluene (BHT) in adipose tissue is described. The concentration of BHT found in the body fat was 0·23 ± 0·15 ppm in 11 residents of the UK and 1·30 ± 0·82 ppm in 12 residents of the USA. These concentrations are of the same order of magnitude as those predicted from the data of Daniel *et al.* (*Fd Cosmet. Toxicol.* 1967, 5, 475) and are 15–80 times less than those found in rats receiving the no-untoward-effect level of BHT (50 mg/kg body weight/day) in their diet.

## Introduction

Butylated hydroxytoluene (3,5-di-*tert*-butyl-4-hydroxytoluene; BHT), used extensively as an antioxidant in food for human consumption, is excreted only slowly following administration to experimental animals. Golder, Ryan & Wright (1962) and Ladomery, Ryan & Wright (1963) showed that rats excreted 33–50% of an oral or parenteral dose of BHT in the urine in 4 days. Daniel & Gage (1965) found that rats excreted 80–90% of an oral dose in 4 days, 25–40% in the urine and the rest in the faeces. The excretion of BHT and its metabolites is delayed by an enterohepatic circulation in the rat.

The rat oxidizes the methyl group on the BHT ring to carboxylic acid and excretes it unbound or as the glucuronide (Wright, Akintonwa, Crowne & Hathway, 1965). The mercapturate conjugate is also excreted in the urine but free BHT is the main component in the faeces (Daniel, Gage & Jones, 1968). BHT given repeatedly to rats accumulates in the fatty tissues. The concentration in subcutaneous adipose tissue reaches a peak in 2 days and then falls in 7 days to a constant level, doses of 500 mg/kg/day producing a final level of 100 ppm BHT in the fat (Gilbert & Golberg, 1965) and doses of 250 mg/kg/day giving a level of 30 ppm (Daniel & Gage, 1965).

The metabolism of BHT by man differs, however, from that in the rat. Free and conjugated BHT-acid is a minor constituent of urine and mercapturate is virtually absent. The bulk is excreted as the glucuronide of a metabolite in which the ring methyl and a methyl of one of the *tert*-butyl groups are oxidized to carboxylic acid, and a methyl of the other *tert*-butyl group is also oxidized, probably to aldehyde (Daniel *et al.* 1968). About 75% of a dose of BHT is excreted by man in the urine and faeces during the first 24 hr but the remainder is excreted more slowly. The 5-day half-life of this second phase of

* Present address: Unilever Research Laboratories, Colworth/Welwyn, Sharnbrook, Beds.
† British Industrial Biological Research Association, Woodmansterne Road, Carshalton, Surrey.

excretion has been attributed to the slow release of BHT or its metabolites from the tissues (Daniel, Gage, Jones & Stevens, 1967). These findings suggest that, dose for dose, BHT will accumulate to a greater extent in the tissues of man than of the rat.

The disadvantages of several methods for the assay of BHT were discussed by Szalkowski & Garber (1962), who described a method based on the separation of BHT from fat or oil by steam distillation followed by colorimetric determination using a di-*o*-anisidine–nitrous acid reagent. Since this reagent gives a colour with other phenolic compounds the emphasis was on the development of a clean-up method to eliminate all reacting substances except BHT. In the method of Daniel & Gage (1965) the lipid containing BHT was extracted from tissues with ether. After evaporation of the ether the mixture was taken up in methanol and the lipid was crystallized out by cooling to −30°C. This left the BHT in solution together with the minimum amount of fat. The BHT was then steam distilled and estimated by the di-*o*-anisidine method. The method required fat samples of 3–5 g and was unsuitable for estimating less than 1 ppm BHT. We describe here a modification of the method of Daniel & Gage (1965) for estimating low concentrations of BHT in fat, and its use in determining the BHT content of human adipose tissue.

## Experimental

Samples of adipose tissue weighing 1–2 g were ground with 10 times their weight of anhydrous sodium sulphate. The resulting powder was extracted overnight in a Soxhlet apparatus with petroleum ether. The extract was dried over sodium sulphate and the solvent was removed by evaporation. The residue was taken up in the minimum volume of 5% (v/v) benzene–petroleum and added slowly to the top of a 30-g silica-gel column made up in the same solvent mixture. This solvent mixture was then passed slowly through the column, from which the BHT was quantitatively eluted in the first 500 ml. The eluate was evaporated and the residue was taken up in 2 ml 50% (v/v) water–methanol. Magnesium oxide (250 mg) was added and the BHT was steam distilled using micro Kjeldahl distillation apparatus. The first 5 ml of distillate contained all the BHT. The colour reaction was carried out using the precautions described by Daniel & Gage (1965) and the colour was extracted into 4 ml chloroform and read in a 3-cm cell at 534 mμ. The recovery of BHT from lipid (arachis oil and human lipid) was better than 90%, and a level of 0·1 ppm could be determined by the method.

## Results

The levels of BHT found in samples of fat extracted from human subcutaneous adipose tissue are shown in Table 1. In six male and five female subjects resident in the area around Birmingham, England, the BHT content of the lipid of this adipose tissue was between 0·01 and 0·49 ppm, with a mean content of 0·23 ppm and standard deviation of 0·15 ppm. In seven male and five female subjects resident in the area around New York, USA, the BHT content of this lipid was between 0·34 and 3·19 ppm, with a mean content of 1·30 ppm and standard deviation of 0·82 ppm. The average age of the British and US residents was 54 and 52 yr respectively. No significant differences in levels of BHT were found between males and females in either country.

## Discussion

The intake of BHT by man has been estimated as 1 mg/head/day in the UK (Gilbert & Martin, 1967) and 2 mg/head/day in the USA (Food and Drug Administration, 1964). Al-

Table 1. *The BHT content of fat extracted from human subcutaneous adipose tissue*

| Patient no. | Sex | Age | Principal cause of death | BHT in fat (ppm) |
|---|---|---|---|---|
| | | | **British Residents** | |
| 1 | M | 60 | Coronary heart disease | 0·18 |
| 2 | M | 42 | Aortic Stenosis | 0·13 |
| 3 | M | 52 | Coronary heart disease | 0·28 |
| 4 | M | 45 | Aortic Stenosis | 0·21 |
| 5 | M | 60 | Coronary heart disease | 0·25 |
| 6 | F | 55 | Rheumatic heart disease | 0·44 |
| 7 | F | 55 | Post operative death | 0·01 |
| 8 | F | 60 | Pulmonary embolus | 0·01 |
| 9 | M | 54 | Coronary heart disease | 0·31 |
| 10 | F | 63 | Coronary heart disease | 0·49 |
| 11 | F | 53 | Coronary heart disease | 0·18 |
| | | | Mean ± SD ..... | 0·23 ± 0·15 |
| | | | **US Residents** | |
| 1 | F | 40 | Hypertensive heart disease | 1·41 |
| 2 | M | 60 | Brain tumour | 2·23 |
| 3 | M | 61 | Pneumonia | 1·36 |
| 4 | F | 48 | Hypertensive heart disease | 1·03 |
| 5 | M | 75 | Brain tumour | 2·06 |
| 6 | M | 66 | Pulmonary embolus | 0·73 |
| 7 | M | 18 | Muscular dystrophy | 0·34 |
| 8 | F | 53 | Post operative death | 0·85 |
| 9 | F | 48 | Coronary heart disease | 3·19 |
| 10 | M | 58 | Coronary heart disease | 0·79 |
| 11 | M | 58 | Carcinoma of bronchus | 0·62 |
| 12 | F | 37 | Pulmonary embolus | 1·00 |
| | | | Mean ± SD ..... | 1·30 ± 0·82 |

SD = Standard deviation

though the conditional acceptable daily intake of BHT is 140 mg/day for a 70-kg man (Joint FAO/WHO Expert Committee on Food Additives, 1967) the provisions of national legislation make the upper limit of intake 4 mg/day (Gilbert & Golberg, 1965). Assuming that 1 mg BHT is eaten each day and that 75% of this is excreted in 24 hr and the remainder is stored in the tissues to be excreted at a rate consistent with a 5-day half-life, it is calculated that BHT will accumulate until the body contains 2 mg. If this is evenly distributed through the total body fat (10 kg) of a 70-kg man (International Commission on Radiological Protection, 1955) it will produce a concentration of 0·2 ppm BHT. This is close to the average concentration found in the adipose tissue of subjects resident in Britain. The concentration produced by a 2 mg/day intake is calculated to be 0·4 ppm which is below that found in the fat of US residents.

The calculation, although not accurate because of the assumptions made, shows that the concentrations of BHT found in human adipose tissue are of the same order of magnitude as those predicted from the data of Daniel *et al.* (1967) on the metabolism of BHT by man.

The results also confirm that on a dose/body weight basis BHT accumulates to a higher concentration in the fatty tissues of man than of the rat. The level of BHT intake in rats which has no detectable untoward effect is 50 mg/kg/day and at this level the concentration

of BHT in adipose-tissue lipid is in the region of 20 ppm (Gilbert & Golberg, 1965), a level approximately 80 and 15 times the concentration found in human lipid in Britain and the USA respectively.

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# QUANTITATION OF THE PROLIFERATIVE RESPONSE OF SMOOTH ENDOPLASMIC RETICULUM TO DIETARY BUTYLATED HYDROXYTOLUENE, AND ITS TOXICOLOGICAL SIGNIFICANCE

D. M. CONNING and T. F. McELLIGOTT*

Industrial Hygiene Research Laboratories, I.C.I. Ltd., Alderley Park, Macclesfield, Cheshire, United Kingdom

Many compounds have been described which induce proliferation of the smooth endoplasmic reticulum (SER) in liver cells and in a majority of instances, it has been shown that such a proliferation is associated with increased production of a drug-metabolising enzyme. Where the compound or its metabolite is an inhibitor of protein synthesis, however, the enzyme response may be defective and its measurement may not, therefore, be a consistent indicator of a response (Meldolesi, 1967).

There has been considerable discussion as to whether the increase of SER should be regarded as a toxicological phenomenon or not and, although it is generally agreed that this represents in part an adaptive response, the normal limits of the response are by no means clear. This problem is unlikely to be resolved until attempts are made to quantitate the response and equate it with the known toxic levels of the compound or drug. Butylated hydroxytoluene (BHT) is an antioxidant known to produce hypertrophy of liver cells (Gilbert and Golberg, 1965), and to be associated with SER production (Lane and Lieber, 1967), and has been used as a model to investigate the relationship between dose levels administered and SER response.

## EXPERIMENTAL PROCEDURE

Three main experiments were undertaken. First, quantitation of the SER in response to feeding BHT in the diet at levels of 5%, 1%, 0.1% and 0.01% for periods of seven to ten days and following the subsequent disappearance of the SER over the next forty to fifty days. Second, the quantitation of the SER response after prolonged feeding of BHT at dose levels of 1%, 0.1% and 0.01% of the diet and the subsequent disappearance of SER following cessation of feeding; and, finally, the feeding of BHT at a level of 1% of the diet for ten days followed by a recovery period of twenty days, and the subsequent feeding for a further ten days to determine whether an initial contact may dispose to a more rapid response.

The animals used were female specific pathogen free Alderley Park rats (Wistar derived), weighing approximately 150–200 grams. The animals were maintained on a standard diet (Oaks of Congleton, Cheshire) to which 18% w/w of maize oil was added. The BHT was incorporated into the test diets as a solution in the maize oil. The animals were killed in groups of two or three throughout the experiment, controls being taken at each time, and sections from various parts of the liver were examined on an electron microscope (AEI EM6B) at a magnification of 15,000 diameter. A grid incorporating 400 points at 1 cm intervals was used to examine photographs taken randomly according to the methods of Weibel *et al.* (1966). Counts were made of all the organelles on which the points were superimposed and the percentage SER of the total organelle count was calculated. It was found that the numbers of mitochondria remained constant and that

* Present address: Department of Pathology, Hôtel Dieu Hospital, Kingston, Ontario, Canada.

if the SER was expressed as a ratio to mitochondria, a more sensitive index was obtained. This we have termed the SER unit.

## RESULTS

The results of short-term feeding (Fig. 1) show that there is a prompt and rapid response to 5%, 1% and 0.1% BHT in the diet. The 0.1% level appears to give a higher count

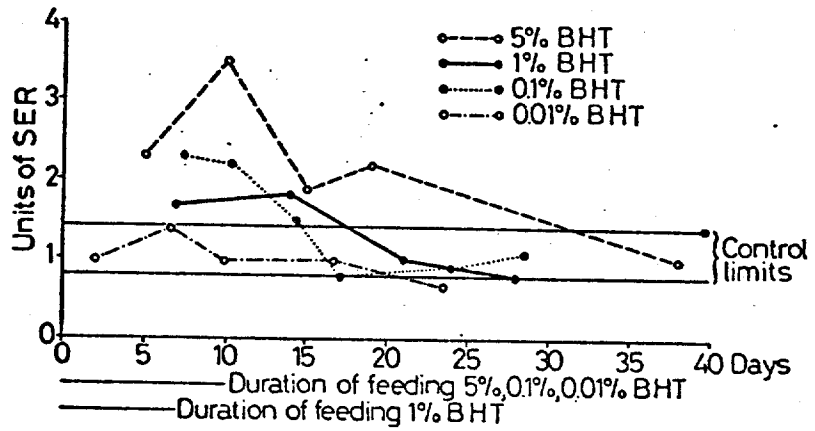


*Fig. 1.* The response of SER to short-term BHT feeding.

than the 1% level, but the difference is not significant. It is noteable that the amount of SER falls off fairly rapidly after cessation of the dietary BHT, except in the case of the 5% level where a much more gradual decline is obtained. The effects of long-term feeding (*i.e.* 30 days, Figure 2) consist of a large progressive response at the 1% level whereas the 0.1% level, though showing a rise in the early stages as was seen in the earlier experiment, remains within normal limits until the end of the feeding period. A slight increase appears at this time, but is not statistically significant. The 0.01% level produces no change. After withdrawal of BHT there is a very marked reduction of the SER, but the animals return to normal within fifteen days.

The rapid removal of the SER is not associated with the normal means of intracellular

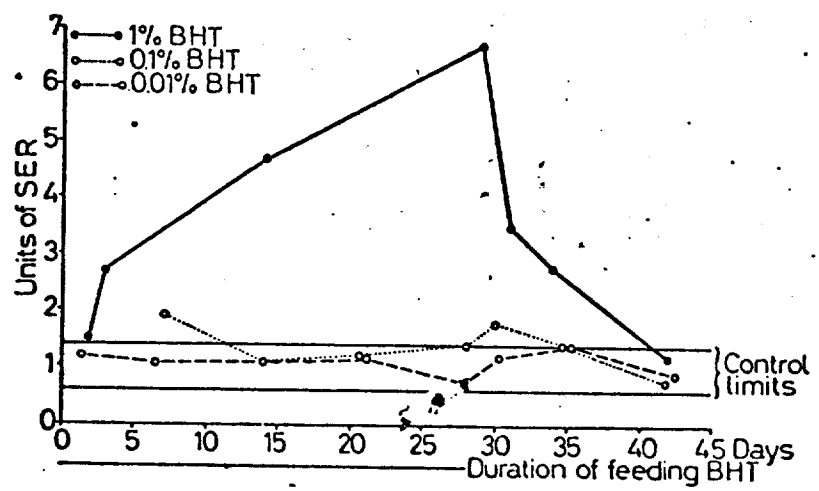


*Fig. 2.* The response of SER to long-term BHT feeding.

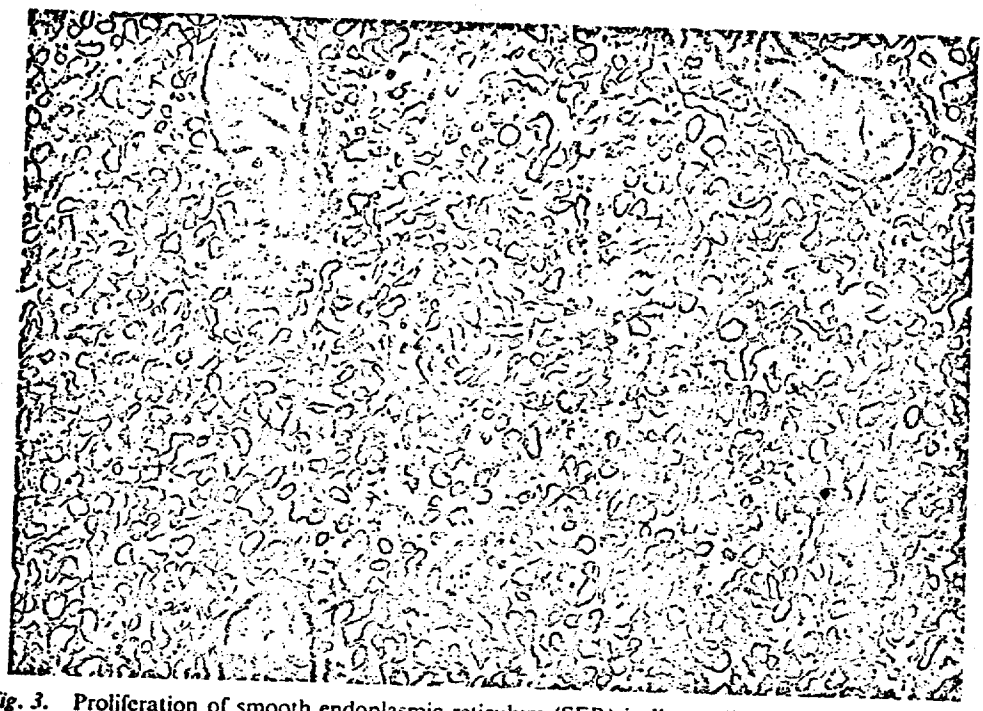

*Fig. 3.* Proliferation of smooth endoplasmic reticulum (SER) in liver cells after feeding 1% BHT for 25 days. × 30,000; reduced for reproduction 20%.

*Fig. 4.* The cell on the left shows dilatation of SER tubules 24 hours after cessation of BHT. × 30,000; reduced 20%.

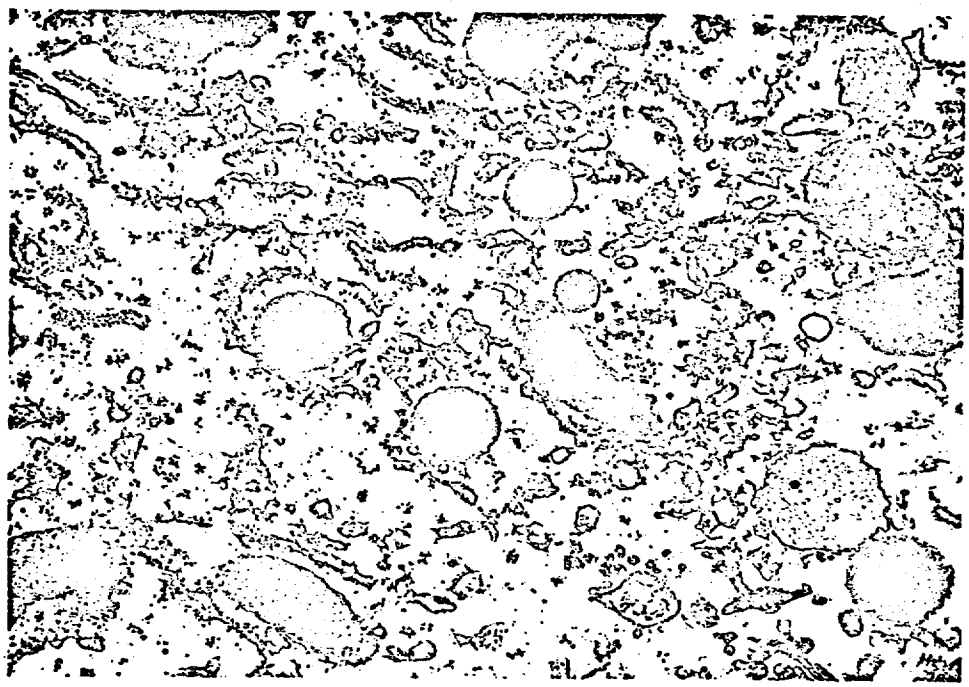

*Fig. 5.* A cell showing wide spacing of endoplasmic reticulum tubules, many showing attached ribosomes, 48 hours after cessation of BHT. Note sparsity of lysosomal response. × 30,000; reduced 20%.

scavenging, such as autophagosome production or increased lysosomal activity. The tubules become dilated and fade away leaving spaces which eventually are filled by other cell constituents. It is possible that some of the SER is converted to rough endoplasmic reticulum (Figs. 3, 4 and 5).

The SER response to a second episode of BHT in the diet twenty days after an initial period of feeding (Fig. 6) is prompt and of no lesser magnitude than that obtained with the initial feed. This would suggest that the response on the second occasion is qualitatively the same as the first and there is, therefore, no evidence of enhanced ability to deal with the BHT or of drug resistance.

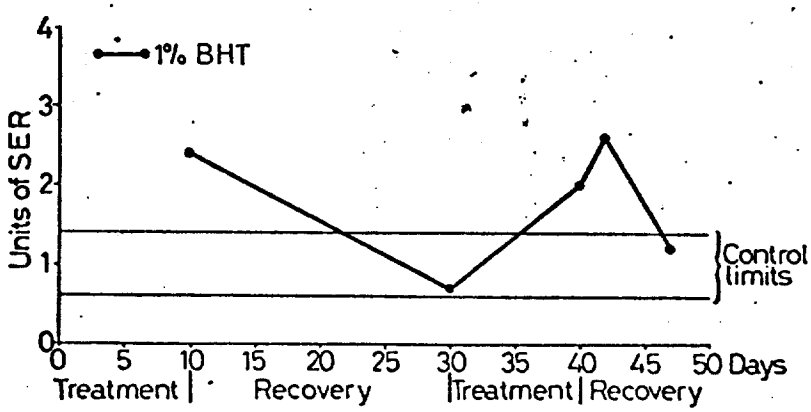


*Fig. 6.* The response of SER to repeated BHT feeding.

## DISCUSSION

The quantitative response of SER to dietary BHT parallels the adaptive enzyme response (Botham *et al.*, 1969) though it is not as sensitive. The results are close enough to suggest, however, that the SER response is fairly accurately measured by the techniques used. Of particular interest is the response to 0.1% BHT in the diet where an initial increase of SER was not maintained. The enzyme response at this dose remained slightly elevated. This may indicate that this dietary level is near the limit of a physiological response as opposed to a toxicological response.

There is insufficient information at present to draw any firm conclusions as to the toxicological significance of SER proliferation in the presence or absence of an adaptive enzyme response, but it is suggested that a solution to this problem will not be obtained until quantitation of the SER response is attempted.

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# The effect of butylated hydroxytoluene, butylated hydroxyanisole and octyl gallate upon liver weight and biphenyl 4-hydroxylase activity in the rat

P. J. CREAVEN, W. H. DAVIES AND R. T. WILLIAMS

When a diet containing butylated hydroxytoluene at levels of 0·01 to 0·5% is fed to growing male rats for 12 days, body weight is not affected, but liver weight is increased. The activity of liver microsomal biphenyl 4-hydroxylase is also increased by these concentrations of butylated hydroxytoluene except the lowest level of 0·01% (100 ppm). Butylated hydroxyanisole at 0·1 and 0·25% of the diet did not affect liver weight or the enzyme activity, but at 0·5% it caused some increase in enzyme activity but not in liver weight. At the level of 0·5%, butylated hydroxytoluene given for a single day caused an increase in liver weight which was observed two days later and persisted for three days after which time the liver weight did not differ from controls. Enzyme activity was not significantly altered by 0·5% butylated hydroxytoluene fed during a single day. Octyl gallate fed at 0·5% of the diet for 9 days did not affect liver weight or enzyme activity. High fat diets with or without 0·01% butylated hydroxytoluene fed for 12 days did not increase liver weight, but did increase biphenyl 4-hydroxylase activity.

IT has been shown by Gilbert & Golberg (1965) that if female rats are given butylated hydroxytoluene (BHT) daily by stomach tube for a prolonged period at a dose of 500 mg/kg, the liver becomes enlarged and there is an increase in the activity of the liver microsomal enzymes which metabolise aminopyrine, hexobarbitone and nitroanisole. Similarly, if the rats are given butylated hydroxyanisole (BHA), there is a less pronounced enlargement of the liver, but no increase in the activity of the above microsomal enzymes. In the present paper we report experiments along similar lines, except that the rats used were males and the BHT and BHA were incorporated into the diet at various concentrations.

The liver microsomal enzyme system examined was that which hydroxylates biphenyl to 4-hydroxybiphenyl and, in a few experiments, that which demethylates 4-methoxybiphenyl. These enzyme systems were chosen because 4-hydroxybiphenyl is easily estimated fluorimetrically (Creaven, Parke & Williams, 1965).

## Experimental

### MATERIALS

BHT (3,5-di-t-butyl-4-hydroxytoluene), m.p. 73° after recrystallisation from light petroleum, BHA (a mixture containing not less than 90% of 3-t-butyl-4-hydroxyanisole, not more than 8% of 2-t-butyl-4-hydroxyanisole and less than 1% of 4-hydroxyanisole), m.p. 52·5° and octyl gallate, m.p. 102·5° (British Drug Houses Ltd., Poole, Dorset).

### ANIMALS AND DIET

Male Wistar albino rats of about 150 g weight (Porton strain; Allington Farm, Porton Down, Salisbury, Wilts) were maintained on 10 g/day of powdered 41B diet (Rank Ltd., Croydon, Surrey) and water *ad lib.* The

From the Department of Biochemistry, St. Mary's Hospital Medical School, London, W.2.

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antioxidants were intimately mixed with the diet when necessary. For high fat diets Walls' pure English lard or Mazola corn oil was used since these were reputed to be free of antioxidants. The diets were fully consumed each day by the rats.

### ENZYME ACTIVITY

The preparation of the 10,000 *g* supernatant of rat liver homogenates, the incubation procedure and the determination of biphenyl 4-hydroxylase activity were made as described by Creaven & others (1965). In the experiments in which 4-methoxybiphenyl demethylase was determined, the method used was that of Creaven, Davies & Williams (unpublished) which is similar to that for biphenyl 4-hydroxylase except that 4-methoxybiphenyl is used as substrate instead of biphenyl.

## Results

### EFFECT OF CONTINUOUS FEEDING OF BHT AND BHA

Table 1 shows that if rats are allowed to eat a BHT-containing diet for 12 days, the weight of the animals is not affected compared with the controls, but the liver weight in relation to body weight is significantly

TABLE 1. THE EFFECT OF FEEDING BHT FOR 12 DAYS UPON THE WEIGHT AND THE BIPHENYL 4-HYDROXYLASE ACTIVITY OF RAT LIVER

| No. of animals | Amount of BHT in diet* % | Increase in body wt. % | Liver wt g/100 g rat | Increase % | P | Biphenyl 4-hydroxylase μmole/g liver/hr | Increase % | P |
|---|---|---|---|---|---|---|---|---|
| 10 | none | 36·4 | 4·0 | — | | 2·33 | — | |
| 9 | 0·01 | 42·8 | 4·8 | 20 | <0·001 | 2·58 | 11 | N.S. |
| 10 | 0·05 | 31·3 | 4·8 | 20 | <0·001 | 2·86 | 23 | 0·05 |
| 10 | 0·10 | 27·7 | 4·6 | 16 | <0·01 | 3·26 | 40 | <0·01 |
| 10 | 0·25 | 42·4 | 5·4 | 36 | <0·001 | 3·93 | 69 | <0·01 |

* 10 g dry rat of powdered diet 418 (Rank Ltd.).
After 12 days the rats were killed, their livers weighed and the 10,000 *g* supernatant of the homogenised liver prepared. The biphenyl 4-hydroxylase activity of each liver was determined fluorimetrically (Creaven & others, 1965).

increased at all levels of BHT (0·01 to 0·25%) in the diet. The activity of microsomal biphenyl 4-hydroxylase is also significantly increased except at the dose level of 0·01% (100 ppm) of BHT.

Table 2 shows the results with BHA diet, and it is clear that neither liver weight nor microsomal enzyme activity, that is biphenyl 4-hydroxylase and 4-methoxybiphenyl demethylase activity, is affected by 0·1 or 0·25% (1,000 or 2,500 ppm) of BHA.

TABLE 2. THE EFFECT OF BHA ON LIVER WEIGHT AND ON CERTAIN LIVER MICROSOMAL ENZYME ACTIVITIES IN THE RAT

| No. of animals | Amount of BHA in diet* % | Liver wt g/100 g rat | Biphenyl 4-hydroxylase activity μmole/g liver/hr | Increase % | P | 4-methoxybiphenyl demethylase μmole/g liver/hr | Increase % | P |
|---|---|---|---|---|---|---|---|---|
| 10 | 0 | 5·8 | 1·67 | — | | 2·70 | — | |
| 6 | 0·1 | 6·0 | 1·33 | −20 | N.S. | 1·98 | −26 | N.S. |
| 6 | 0·25 | 5·7 | 1·74 | +4 | N.S. | 2·37 | −12 | N.S. |

* Details as in Table 1.
After 12 days the animals were killed. The livers were weighed and biphenyl 4-hydroxylase and 4-methoxybiphenyl demethylase activity of the 10,000 *g* supernatant of each homogenised liver were determined fluorimetrically.

EFFECT OF BHT FEEDING FOR DIFFERENT PERIODS

BHT was incorporated in the diet at the level of 0·5% (5,000 ppm). After feeding this diet for a single day before killing the animals, there was no significant effect upon liver weight or biphenyl 4-hydroxylase activity (see Table 3). However, if this BHT-containing diet was fed for two or more days before killing, the liver weight and the microsomal enzyme activity were significantly increased.

TABLE 3. EFFECT OF FEEDING BHT FOR DIFFERENT PERIODS UP TO 6 DAYS UPON THE WEIGHT AND BIPHENYL 4-HYDROXYLASE ACTIVITY OF RAT LIVER

| No. of animals | Period over which BHT 5,000 ppm (0·5%) was fed in diet* days | Liver wt g/100 g rat | Increase % | P | Biphenyl 4-hydroxylase units/g liver/hr | Increase % | P |
|---|---|---|---|---|---|---|---|
| 5 | 0 | 5·0 | — | | 1·14 | — | |
| 5 | 1 | 5·5 | 10 | N.S. | 1·28 | 11 | N.S. |
| 5 | 2 | 6·3 | 26 | <0·001 | 1·59 | 39 | <0·001 |
| 5 | 3 | 6·6 | 32 | <0·001 | 1·90 | 67 | <0·001 |
| 5 | 4 | 6·0 | 20 | <0·001 | 2·53 | 122 | <0·001 |
| 5 | 5 | 6·3 | 26 | <0·001 | 2·59 | 126 | <0·001 |
| 5 | 6 | 6·5 | 30 | <0·001 | 2·45 | 115 | <0·001 |

* Diet as in Table 1.
The various groups were given the BHT diet for 1–6 days so that all groups except the control received the BHT-containing diet on the 6th day and were then killed at the end of the 7th day. The livers were weighed and biphenyl 4-hydroxylase activity determined.

If, however, the BHT-containing diet was fed for one day only, and then the animals were transferred to a BHT-free diet for varying periods up to 8 days, then, as Table 4 shows, one day on the diet containing 0·5% BHT significantly affects the liver weight but not the biphenyl 4-hydroxylase activity. The effect on liver weight, however, does not appear until the second day after the day on the BHT-containing diet.

TABLE 4. THE EFFECT OF TIME AFTER A SINGLE DOSE OF BHT UPON LIVER WEIGHT AND BIPHENYL 4-HYDROXYLASE ACTIVITY IN THE RAT

| No. of animals | No. of days alive after 1 day with BHT 5,000 ppm (0·5%) in diet* | Liver wt g/100 g rat | Increase % | P | Biphenyl 4-hydroxylase units/g liver/hr | Increase % | P |
|---|---|---|---|---|---|---|---|
| 5 | control, no BHT | 5·4 | — | | 1·97 | — | |
| 5 | 1 | 6·0 | 11 | N.S. | 2·21 | 12 | N.S. |
| 5 | 2 | 7·3 | 35 | <0·001 | 2·17 | 10 | N.S. |
| 5 | 3 | 8·3 | 54 | <0·001 | 2·25 | 14 | N.S. |
| 5 | 5 | 6·7 | 24 | 0·05 | 2·05 | 4 | N.S. |
| 5 | 6 | 5·6 | 4 | N.S. | 1·99 | 1 | N.S. |
| 5 | 7 | 6·0 | 11 | N.S. | 2·12 | 8 | N.S. |
| 5 | 8 | 5·5 | 2 | N.S. | 2·05 | 4 | N.S. |

* Diet as in Table 1.
Each group received this BHT diet for only 1 day but on different days (as indicated in the table) before they were killed.
All the rats were killed on the 9th day, their livers weighed and biphenyl 4-hydroxylase activity determined fluorometrically.

Table 4 also shows that the effect of one day on 0·5% BHT (during which time each rat would have consumed 50 mg of BHT) persists for about 4 days and after that time the liver weight returns to control values.

These experiments show that the liver weight is more sensitive to BHT than is biphenyl 4-hydroxylase activity.

EFFECT OF HIGH FAT DIETS

It has been reported that BHT given with a high fat diet might produce toxic phenomena not observed with a low fat diet (Johnson & Howell,

1961; but see Brown, Johnson & O'Halloran, 1959). Table 5 shows that high fat diets alone for 12 days cause significant increases in biphenyl 4-hydroxylase activity and these increases are no greater when 0·01%

TABLE 5. EFFECT OF A HIGH FAT DIET WITH OR WITHOUT BHT ON THE LIVER WEIGHT AND BIPHENYL 4-HYDROXYLASE ACTIVITY IN THE RAT

| No. of animals | Diet* | BHT in diet % | Increase in body wt % | Liver wt g/100 g rat | Increase % | Biphenyl 4-hydroxylase μmole/g liver/hr | Increase % | P |
|---|---|---|---|---|---|---|---|---|
| 10 | control | 0 | 124 | 5·8 | — | 1·56 | — | |
| 5 | + 20% lard | 0 | 92 | 5·3 | −9 | 2·48 | 59 | <0·001 |
| 5 | + 20% lard | 0·01 | 113 | 5·1 | −12 | 2·68 | 72 | <0·001 |
| 5 | + 20% corn oil | 0 | 107 | 4·8 | −17 | 2·73 | 75 | <0·001 |
| 5 | + 20% corn oil | 0·01 | 104 | 4·8 | −17 | 2·78 | 78 | <0·001 |

* *Controls:* Diet 41B (Rank Ltd.) 15 g/day.

*Experimental animals:* Diet 41B (12 g) containing Walls' pure English lard (3 g) or Mazola corn oil (3 g). BHT at 100 ppm (0·01%) was added as indicated in the Table.

Each group was given the diet indicated in the table for 12 days when all the rats were killed. Body and liver weights were recorded and the biphenyl 4-hydroxylase activity of the liver determined.

BHT is incorporated into the high fat diet. None of the diets used in Table 5 increase the ratio, liver weight/body weight. This ratio, if anything, fell on the high fat diets. The fall in this ratio, however, does not account for the increase in enzyme activity.

## COMPARISON OF THE ANTIOXIDANTS

Table 6 records an experiment made on five groups of rats simultaneously. It will be seen that at a level of 0·5% in the diet, the antioxidant, octyl gallate, does not affect the liver weight or the biphenyl 4-hydroxylase when fed for 9 days. Under the same conditions BHA does not affect the liver weight but it does significantly increase the

TABLE 6. THE EFFECT OF BHT, BHA, OCTYL GALLATE AND A MIXTURE OF THESE ANTIOXIDANTS UPON LIVER WEIGHT AND LIVER BIPHENYL 4-HYDROXYLASE AND 4-METHOXYBIPHENYL DEMETHYLASE OF THE RAT

| No. of animals | Antioxidant added % of diet* | Liver wt g/100 g rat | Increase % | P | Biphenyl 4-hydroxylase μmole/g liver/hr | Increase % | P | 4-Methoxybiphenyl demethylase μmole/g liver/hr | Increase % |
|---|---|---|---|---|---|---|---|---|---|
| 8 | none | 5·6 | — | | 1·93 | — | | 2·23 | — |
| 8 | BHT 0·5 | 7·3 | 30 | <0·001 | 4·14 | 114 | <0·001 | 4·18 | 87 |
| 8 | BHA 0·5 | 6·3 | 12·5 | N.S. | 2·81 | 46 | <0·001 | — | — |
| 6 | Octyl gallate 0·5 | 5·1 | none | | 2·01 | 4 | N.S. | — | — |
| 6 | BHT 0·19, BHA 0·19, Octyl gallate 0·125 | 7·1 | 27 | <0·01 | 3·33 | 72 | <0·001 | — | — |

* Diet as in Table 1.

After 9 days on the diets the rats were killed, the liver wt/body wt determined. Biphenyl 4-hydroxylase activity was determined. 4-Methoxybiphenyl demethylase activity was determined in the controls and the group receiving BHT only.

P<0·001

enzyme activity. However, BHT affects all the parameters, the liver weight, biphenyl 4-hydroxylase and 4-methoxybiphenyl demethylase. Table 6 also shows the effect of a mixture of the antioxidants incorporated at the level of 0·5% of the diet. This mixture consisted of BHT (1,900 ppm), BHA (1,900 ppm) and octyl gallate (1,250 ppm). Liver weight

and biphenyl 4-hydroxylase activity were increased by this mixture, and the figures in Table 6 suggest the effect may have been due to its BHT content.

## Discussion

The administration of certain drugs and other foreign compounds to rats and mice is known to stimulate the activity of the liver microsomal enzymes which metabolise foreign compounds (see Conney & Burns, 1962). The significance of this stimulation, as far as the well-being of the animal is concerned, is not yet fully understood, for under some circumstances it may be advantageous, whereas under others it may not. The stimulation of enzymes which destroy and detoxicate deleterious substances in the body is obviously an advantage, but the stimulation of enzymes which convert foreign compounds into toxic agents is not (see Williams, 1963).

That the administration of certain foreign compounds to animals such as rats and mice causes liver enlargement has been known for some time as a routine observation in toxicological testing (see Gilbert & Golberg, 1965), but its significance in several instances is not really understood. Gilbert & Golberg (1965) have put forward the view that this enlargement of the liver may not be a toxic manifestation but a physiological response of the liver to certain foreign compounds which is accompanied by an increase in the activity of certain liver enzymes concerned in the metabolism of these compounds. Usually relatively large doses of these compounds are needed to produce this response. Liver enlargement in rats can be obtained even with sodium benzoate if the dose is large enough, that is 8% of the diet (Deuel, Alfin-Slater, Weil & Smyth, 1954). With BHT, as shown in this paper, significant enlargement of the liver in the rat can be produced by a diet containing 100 ppm (1 mg/day/rat) of BHT (see Table 1), although this amount does not significantly increase the biphenyl 4-hydroxylase activity of the liver. Neither BHA nor octyl gallate has this effect upon liver weight even when they are fed at 5,000 ppm for 9 days, although at this level BHA does increase the enzyme activity (see Table 6). If liver enlargement has any toxicological significance then it would appear that either BHA or octyl gallate is preferable to BHT. At the present time our knowledge is insufficient to decide whether enlargement of the liver by substances like BHT is a manifestation of toxicity or not, and whether such an enlargement is peculiar to rats and mice or is a general phenomenon.

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# The Metabolism of 3:5-Di-*tert.*-butyl-4-hydroxytoluene and 3:5-Di-*tert.*-butyl-4-hydroxybenzoic Acid in the Rabbit

By J. C. DACRE

*Toxicology Research Unit of the Medical Research Council of New Zealand, Medical School, University of Otago, Dunedin, New Zealand*

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The compound known commercially as butylated hydroxytoluene (3:5-di-*tert.*-butyl-4-hydroxytoluene) is an antioxidant developed initially for use with petrol, petroleum products and rubber. Dunn, Marx, Weir & Kraybill (1951) found butylated hydroxytoluene to be an effective antioxidant for animal fats at a concentration of 0·01%, and legislative approval for its addition to fats and foods containing fats intended for human consumption has already been given in eight countries including U.S.A., Denmark, Sweden and the United Kingdom. The toxicity of the compound to rabbits, rats, guinea pigs, cats and dogs was investigated by Deichmann, Clemmer, Rakoczy & Bianchino (1955), who found that when it was given orally to rabbits, there was an increased excretion of both glucuronide and ethereal sulphate in the urine. The causes of the toxic and lethal effects in rabbits given large doses of butylated hydroxytoluene were investigated by Denz & Llaurado (1957) and some aspects of its chronic toxicity in rats were studied by Brown, Johnson & O'Halloran (1959).

It will be shown in this paper that 3:5-di-*tert.*-butyl-4-hydroxybenzoic acid is a metabolite of butylated hydroxytoluene in both the free and conjugated forms. The metabolism of this acid and of the antioxidant butylated hydroxytoluene has been investigated in the rabbit and the major metabolites excreted in the urine have been isolated and characterized. A summary of these results has been published (Dacre, 1960).

## EXPERIMENTAL

*Animals, diet and dosage.* The rabbits used were does of the New Zealand white strain; weighing 1·33–2·0 kg. They were maintained on a standard pellet diet (wheat, 40%; pollard, 33%; bran, 27%) and during experiments were kept singly in metabolism cages designed to permit the separate collection of urine and faeces. For the isolation of metabolites in quantity, rabbits were maintained on a modified diet (wheat, 35%; pollard, 20%; meat meal, 15%; bran, 10%; maize meal, 10%; milk powder, 7·5%; bone meal, 1·7%; cod-liver oil, 0·8%). Water and food were supplied *ad lib.* and the urine was collected daily and stored at −10°. In the initial experiments, single doses of 0·8 g. of butylated hydroxytoluene (BHT)/rabbit were dissolved in 7·2 ml. of olive oil and administered by stomach tube. In later experiments, doses of 0·5 and 1·0 g. in 5 ml. of olive oil/rabbit or 0·8 g. of BHT in 4 ml. of olive oil/kg. body wt. of the

animals were given. Three consecutive daily doses of 0·28 and 0·65 g. of 3:5-di-*tert.*-butyl-4-hydroxybenzoic acid in 5 and 10 ml. respectively of olive oil were similarly administered to a rabbit. The animals usually refused food during the 24 hr. after receiving single doses of BHT and they died between 4 and 10 days after the discontinuation of the repeated doses. No obvious ill-effects were observed in the rabbit receiving the three daily doses of the acid, apart from mild anorexia.

*Materials.* BHT (Ionol) was purchased from the Shell Company of New Zealand and bacterial β-glucuronidase from the Sigma Chemical Co., St Louis, Mo., U.S.A. 2:4-Dihydroxytoluene was given by Dr S. Dagley, University of Leeds; 3:5-di-*tert.*-butyl-4-hydroxybenzoic acid and 3:5-di-*tert.*-butyl-4-hydroxybenzyl alcohol, for mixed m.p. and determination of spectra, were given by Dr C. R. Yohe, Section of Coal Chemistry, Illinois State Geological Survey Division, Urbana.

## *Preparation of reference compounds*

Combustion analyses were carried out by Dr A. D. Campbell, Chemistry Department, University of Otago. All methoxy (OMe) values are corrected (see Campbell & Cuttlebergh, 1959). All melting points are uncorrected.

*Derivatives of butylated hydroxytoluene.* 3:5-Di-*tert.*-butyl-4-hydroxybenzaldehyde was prepared by bromine oxidation of BHT dissolved in *tert.*-butanol according to Coppinger & Campbell (1953); 3:5-di-*tert.*-butyl-4-hydroxybenzoic acid was prepared from the aldehyde by the Cannizzaro reaction (10–15% yield) and 3:5-di-*tert.*-butyl-4-hydroxybenzyl alcohol by lithium aluminium hydride reduction of the aldehyde according to Yohe *et al.* (1956). The acid was also prepared in greater yield from the urine of rabbits which had been given BHT orally.

*3:5-Di-*tert.*-butyl-4-hydroxybenzoic acid from rabbit urine.* Urine (250 ml., pH 5·65, containing approx. 16 μmoles of total phenols/ml.) from rabbits given BHT, was incubated with β-glucuronidase powder (0·2 g., about 10 000 units) with intermittent shaking in a water bath at 37° for 24–30 hr. The crystalline material which separated was collected, dried and extracted with peroxide-free ether (2 × 200 ml.) to remove the enzyme residue. Removal of the solvent *in vacuo* yielded 0·54 g. of colourless crystals, m.p. 185–195°. Extraction of the residual urine with peroxide-free ether (2 × 200 ml.) yielded a further 69 mg. (total recovery 61%). One recrystallization from light petroleum (b.p. 60–80°) or vacuum sublimation (110–120°/0·05 mm. Hg) gave colourless plates of 3:5-di-*tert.*-butyl-4-hydroxybenzoic acid, m.p. 210°, unchanged by admixture with an authentic sample (Found: C, 71·75; H, 8·9; mol. wt. 228. Calc. for $C_{15}H_{22}O_3$: C, 72·0; H, 8·8%; mol.wt. 250). Yohe *et al.* (1956) record m.p. 218° for this acid. When determined in sealed tubes from a cold electrically-heated block, Yohe's sample, the sample described above and a mixture of the two all melted at 209–210°.

*Methyl 3:5-di-*tert.*-butyl-4-hydroxybenzoate.* 3:5-Di-*tert.*-butyl-4-hydroxybenzoic acid (100 mg.) was dissolved in dry ether (100 ml.) and diazomethane (about 2 g.) in dry ether was slowly added with intermittent shaking. The reaction mixture was kept for 4 hr. and the ether was then removed *in vacuo* to yield dull yellowish crystalline plates of m.p. 163–165° (82 mg., 78%). Recrystallization from light petroleum (b.p. 60–80°) gave large colourless plates, m.p. 164–165° with sublimation, of *methyl 3:5-di-*tert.*-butyl-4-hydroxybenzoate* (Found: C, 72·75; H, 8·9; OMe, 11·2. $C_{16}H_{24}O_3$ requires C, 72·7; H, 9·1; OMe, 11·7%).

*3:5-Di-*tert.*-butyl-2:4-dihydroxytoluene.* 2:4-Dihydroxytoluene (1·5 g., m.p. 94–95°) was dissolved in dry benzene (50 ml.) and conc. $H_2SO_4$ (0·1 ml.) added. Dry isobutylene was bubbled through the stirred mixture at 50–60° for 4 hr. The benzene soln. was shaken out with 2N-NaOH (2 × 25 ml.) and then with water. The benzene was removed *in vacuo* to give almost colourless crystals (1·44 g., 50%) of m.p. 145–146°. Repeated sublimation *in vacuo* below 100° yielded large colourless rods, m.p. 146–147°, of *3:5-di-*tert.*-butyl-2:4-dihydroxytoluene* (Found: C, 76·4; H, 10·25. $C_{15}H_{24}O_2$ requires C, 76·2; H, 10·2%).

## *Analytical methods*

All spectrophotometric measurements were made with a model SP. 600 spectrophotometer (Unicam Instruments Ltd., London).

*Free and total phenols.* Phenols were determined on diluted rabbit urine, both before and after acid hydrolysis, by the method of Folin & Ciocalteu (1927). The final concentration of phenol was adjusted to 0·3–1·5 mM and the developed colour was measured at 700 Å. The calibration curves of extinction against concentration for phenol, quinol, 2:4-dihydroxytoluene, 3:5-di-*tert.*-butyl-2:4-dihydroxytoluene and 3:5-di-*tert.*-butyl-4-hydroxybenzoic acid gave the same line for the range 0·3–1·5 mM. BHT does not give a blue colour with the Folin & Ciocalteu reagent. When 3:5-di-*tert.*-butyl-4-hydroxybenzoyl β-D-glucosiduronic acid (17·5, 35 and 70 μmoles/ml.) was added to urine, the mean recoveries, determined as free phenols, were 101·3, 98·9 and 99·0% respectively. This glucuronide therefore would contribute to free phenol values.

*Non-conjugable phenols.* These were determined by extracting urine at pH 5·5–6·5 with peroxide-free ether and analysing the evaporated extracts by the Folin & Ciocalteu method.

*Glucuronides.* (*a*) Total glucuronides. The naphtharesorcinol method, as modified by Bray, Humphris, Thorpe, White & Wood (1952), was used for the estimation of total glucosiduronic acid. The naphtharesorcinol reagent (aq. 0·4%) was prepared according to Fishman & Green (1955). Urine was diluted to give a glucuronide concentration between 0·5 and 2·0 mM and readings were made at 600 Å. A calibration curve was prepared with 2-*tert.*-butyl-4-methoxyphenyl β-D-glucosiduronic acid (Dacre, Denz & Kennedy, 1956).

(*b*) Ester glucuronides. The Shaffer & Hartmann (1921) copper reduction method as described by Bray & Thorpe (1954) was used for the estimation of glucosiduronic acid liberated by alkaline hydrolysis of ester-type glucuronides.

*Hippuric acids.* Glycine conjugates in diluted rabbit urine were estimated by the micro-method of Gaffney, Schreier, DiFerrante & Altman (1951) as modified by El Masry, Smith & Williams (1953). The yellow azlactone formed from the hippuric acid and *p*-dimethylamino benzaldehyde was measured at 460 Å. The calibration curve prepared with hippuric acid was linear over the range 0–0·56 mM.

*Mercapturic acids.* The method of Stekol (1936) (iodine consumption after hydrolysis by alkali) as modified by Parke & Williams (1951) was used.

*Ethereal sulphates.* These were determined by Sperber's (1948) modification of the turbidimetric method. The

calibration curve was made with $K_2SO_4$ and readings were taken in the range 0–1·0 mg. of $SO_3$.

### *Paper chromatography*

Phenolic compounds and their metabolites were separated on Whatman no. 1 filter-paper sheets by the descending method with the solvent systems described in Tables 1 and 2. The solvent front was run for 30–40 cm. The compounds were detected and identified on paper with the following spray reagents (all prepared on a w/v basis). (1) Gibb's reagent: a solution of 2:6-dichloro-*p*-benzoquinone-4-chloroimine (0·1%) in ethanol (95%) followed by a solution of sodium tetraborate (aq. 2·0%, pH 9·21). (2) Pauly reagent: a freshly prepared diazotized sulphanilic acid soln. (0·3%) in HCl (8%) followed by a solution of $Na_2CO_3$ (aq. 20%). (3) Molybdophosphoric acid–ammonia reagent: a solution of dodecamolybdophosphoric acid (0·5%) in ethanol (95%) followed immediately by exposure to conc. ammonia fumes (Mitchell, 1957). (4) Naphtharesorcinol–trichloroacetic acid reagent: this was as described by Elliott, Parke & Williams (1959); the reagent does not react on paper with acetylated–methylated glucuronides. (5) Altman reagent: a freshly prepared solution of *p*-dimethylaminobenzaldehyde (4%) in acetic anhydride (containing a few crystals of sodium acetate), followed by heating at 130–150° for 1–2 min. (Gaffney *et al.* 1954).

*Glycine.* This was identified by the method of Consden, Gordon & Martin (1944). The solvent system used was phenol saturated with water (3% ammonia added in the tank) and the acid was detected with a ninhydrin spray reagent (0·1% in butan-1-ol).

### *Spectrophotometry*

Ultraviolet-absorption spectra were measured with a model DK2 recording spectrophotometer (Beckman Instruments Inc., Calif., U.S.A.). The u.v.-absorption data for the derivatives and metabolites of BHT are set out in Tables 1 and 2 respectively. Infrared spectra of BHT and its metabolites were measured with a model IR2 Beckman recording spectrophotometer in KBr disks.

### *Metabolites of butylated hydroxytoluene*

Urine from rabbits given BHT had pH 5·8–6·3 and reduced Fehling and Benedict reagents. It gave no colour reaction with aq. or ethanolic $FeCl_3$ soln. and did not react with 2:4-dinitrophenylhydrazine. By paper chromatography of the urine, compounds A, B, C and D were detected and some of their properties are shown in Table 2. Compound A was detected in ether extracts of urine as collected and B, C and D in ether extracts of urine at pH 2–3. BHT was not detected, and in some urines free glucuronic acid and glucuronolactone were present.

*Isolation of 3:5-di-*tert.*-butyl-4-hydroxybenzoic acid.* Urine (250 ml., pH 6·0) from a bulked supply collected over 7 days after a rabbit was given the first of five repeated daily doses of BHT (1 g.) was extracted with peroxide-free ether (4 × 100 ml.). The combined extracts were reduced *in vacuo* to yield a brownish-yellow crystalline deposit, m.p. 180–185° (0·32 g., 10·8% of the dose). This material was redissolved in ether (100 ml.) and then shaken out with $NaHCO_3$ soln. (aq. 5%); the acid was liberated from the bicarbonate solution and collected by extraction with ether (2 × 100 ml.). Repeated recrystallization from light petroleum (b.p. 60–80°) gave large colourless plates, m.p. 205–209° (0·2 g.), which was not depressed when admixed with authentic 3:5-di-*tert.*-butyl-4-hydroxybenzoic acid (Found: C, 72·4; H, 8·4%).

*Preparation of the ether-soluble glucuronide fractions.* Urine (200 ml.) from which the non-conjugated phenols had been removed (see above) was adjusted to pH 1·5–2·0 with dil. $H_2SO_4$ and extracted again with peroxide-free ether (3 × 200 ml.). The combined extracts were reduced *in vacuo* to a yellow–brown gum (1·0 g.), which gave a strongly positive test for both glucuronic acid and phenol (fraction 1). Paper chromatography showed the presence of glucuronides B and C as well as trace amounts of the glycine conjugate (compound D). The residual urine contained about 10% of the original glucuronide-reacting material. Continuous ether extraction removed this almost completely as well as the glycine conjugate. The solvent was removed *in vacuo* to yield a yellow–brown gum (60 mg.) (fraction 2). Paper chromatography showed the presence of compounds B, C and D.

*Enzymic hydrolyses with β-glucuronidase.* The β-glucuronidase powder was examined for hippuricase (aminoacylase 1) activity by the methods of Ellis & Walker (1942), hippuric acid being used as the substrate. At pH 3·1, 4·6 and 6·1 no free glycine was found after incubation for 2, 24 and 48 hr. with β-glucuronidase at 37°. The manufacturers stated that the enzyme preparation had no sulphatase activity on *p*-nitrophenyl sulphate.

(*a*) Rabbit urine. Urine (150 ml., pH 6·45), penicillin (10 000 units, Glaxo Crystapen) and β-glucuronidase powder (0·1 g., 5000 units) were incubated at 37° in a slow stream of $N_2$. After 6 hr. more β-glucuronidase powder (0·1 g.) was added and the incubation continued for a further 24 hr. with intermittent shaking. The crystalline ppt. was collected, dried and extracted with peroxide-free ether (2 × 100 ml.). Removal of the ether *in vacuo* gave an almost colourless crystalline product (0·22 g.), m.p. 170°, which was shown by paper chromatography with solvent system A (Table 1) to contain 3:5-di-*tert.*-butyl-4-hydroxybenzoic acid ($R_F$ 0·45) and an unidentified substance ($R_F$ 0·6). This was later shown to be identical with β-(3-*tert.*-butyl-2-hydroxy-5-methylphenyl)-ββ-dimethylethanol, the aglycone of glucuronide C. The same compounds were identified in ether extracts of the residual urine.

(*b*) Ether-soluble glucuronide extract (fraction 1). Glucuronide gum (110 mg.) dissolved in 0·2M-sodium acetate buffer (25 ml., pH 4·5) was incubated with β-glucuronidase (13 mg., 660 units) at 37°. Samples were removed and the free glucuronic acid was determined by the cerimetric method of Levvy (1956). After 1 hr. 72% of the glucuronides were hydrolysed and 77% after 7 hr. In buffer of pH 5·5, 90% of the material was hydrolysed after 20 hr.

Glucuronide gum (0·5 g.) was incubated in acetate buffer (100 ml., pH 5·6) with β-glucuronidase powder (0·1 g., 5000 units) for 24 hr. at 37°. The crystals which separated (185 mg.) had m.p. 170–175°. Paper chromatography with solvent system A (Table 1) showed the same two compounds with $R_F$ 0·45 and 0·6 as described in (*a*) above. Recrystallization from light petroleum (b.p. 60–80°) gave 3:5-di-*tert.*-butyl-4-hydroxybenzoic acid, m.p. 210° unchanged by admixture with the authentic acid, and having the same u.v. spectrum in 95% (w/v) ethanol (Found: C, 72·6; H, 8·6%).

**Table 1.** *$R_F$ values, colour reactions and absorption spectra of butylated hydroxytoluene and its derivatives*

Solvent mixtures (by vol., upper phases): A, *cyclo*hexane–methanol (2:1); B, *cyclo*hexane–benzene–methanol (20:10:1); C, ethyl acetate–pyridine–water (2:1:2). Colours of spots: B, blue; G, green; Gy, grey; M, magenta; O, orange; Y, yellow; d, deep; p, pale. Colour on keeping is given in parentheses.

| | $R_F$ value in solvent system | | | Colour with reagent | | | Light-absorption in 95% ethanol | |
|---|---|---|---|---|---|---|---|---|
| | A | B | C | Gibbs | Pauly | Molybdo-phosphate | $\lambda_{max.}$ (mμ) | $\epsilon_{max.}$ |
| 3:5-Di-*tert.*-butyl-4-hydroxytoluene (BHT) | 0·85 | 0·9 | 0·9–1·0 | pY* | None | pGB | 208, 273, 284 ~ † | 8030, 2200 |
| 3:5-Di-*tert.*-butyl-4-hydroxybenzoic acid | 0·45–0·5 | 0·8 | 0·9 | M | YO | YG | 214, 290 | 25500, 13145 |
| 3:5-Di-*tert.*-butyl-4-hydroxybenzaldehyde | 0·65 | 0·8 | — | pM | None | pYG | 227·5, 290 | 18720, 10615 |
| 3:5-Di-*tert.*-butyl-4-hydroxybenzyl alcohol | 0·4 | 0·8 | 0·9–1·0 | M | Y | YG | 240, 273·5, 260·5, 281 ~ | — |
| 3:5-Di-*tert.*-butyl-2:4-dihydroxytoluene | 0·7 | 0·9 | 0·9–1·0 | None (pY) | — | pGyB | 224, 282·5, 286 ~ | 7180, 3260 |
| Methyl 3:5-di-*tert.*-butyl-4-hydroxybenzoate | 0·8 | 0·85 | — | None (dB) | None | None | 291 | 13440 |
| β-(3-*tert.*-Butyl-2-hydroxy-5-methylphenyl)-ββ-dimethylethanol | 0·6 | 0·8–0·85 | 0·9–1·0 | pY | None | pB | 233 | 2790 |

* Colour is intensified on heating at 100° for 5 min.
† See also Tollenaar & Vos (1956); Phillips & Hinkel (1957).

**Table 2.** *$R_F$ values, colour reactions and light-absorption of metabolites of butylated hydroxytoluene*

Solvent mixtures (by vol., upper phases): C, ethyl acetate–pyridine–water (2:1:2); D, lower phase of ethylene dichloride–*tert.*-butanol–acetic acid–water (75:25:30:70); E, butan-1-ol–water (1:1); F, butan-1-ol–2N-ammonia (1:1). Colours of spots: B, blue; M, magenta; Y, yellow; d, deep; p, pale. Colour on keeping is given in parentheses.

| | $R_F$ value in solvent system | | | | Colour with reagent | | Light-absorption in methanol |
|---|---|---|---|---|---|---|---|
| | C | D | E | F | Gibbs | Naphtha-resorcinol | $\lambda_{max.}$ (mμ) |
| Compound A (unconjugated phenol) | 0·9 | — | 0·95–1·0 | 0·8 | pM | None | 212, 258 |
| Compound B (ester glucuronide) | 0·6 | 0·75–0·8 | 0·8 | 0·5–0·55 | Y(M) | dB | 210, 266·5 |
| Compound C (ether glucuronide) | 0·45–0·5 | 0·6 –0·65 | 0·7 | 0·2 | B | dB | 210, 277 |
| Compound D* (glycine conjugate) | 0·3 –0·35 | 0·25 | 0·5 –0·55 | 0·1 | B(dB) | None | 211, 261·5, 266 ~ |

* Orange–yellow with the Altman reagent.

*Isolation of methyl* [*β*-(3-tert.-*butyl*-2-*hydroxy*-5-*methylphenyl*)-*ββ*-*dimethylethyl* 2:3:4-*tri*-O-*acetyl*-*β*-D-*glucopyranosid*]*uronate*. From urine (500 ml.) of rabbits given BHT, the glucuronide gum from the basic lead acetate ppt. at pH 8 was prepared and methylated with diazomethane and then acetylated as described by Kamil, Smith & Williams (1951). Trituration of the dark gummy product with absolute ethanol produced large colourless plates (0·66 g.), m.p. 160–162°. Recrystallization from ethanol gave sheaths of long, fine colourless plates of *methyl* [*β*-(3-tert.-*butyl*-2-*hydroxy*-5-*methylphenyl*)-*ββ*-*dimethylethyl* 2:3:4-*tri*-O-*acetyl*-*β*-D-*glucopyranosid*]*uronate*, m.p. 167·5° (decomp.), $[\alpha]_D^{17.5}$ −45·0° in $CHCl_3$ (*c*, 1·0); light-absorption max., 208 and 279 mμ (ε 10 400 and 2500 respectively) in 95% ethanol (Found: C, 60·7, 61·0; H, 7·2, 7·2; OMe, 5·7, 5·2; $CH_3$·CO, 24·2, 25·6; mol.wt., 541. $C_{23}H_{40}O_{11}$ requires C, 60·9; H, 7·3; OMe, 5·6; $3CH_3$·CO, 23·4%; mol.wt., 552).

Ether-soluble glucuronide gum (fraction 1) (0·5 g.), when methylated, acetylated and triturated as described above, yielded large colourless plates, m.p. 160° (0·2 g.). Recrystallization from *tert.*-butanol gave long flat colourless plates, or flat colourless prisms from methanol, m.p. 165·5° (decomp.) which was not depressed on admixture with the acetylated-methylated glucuronide from the lead acetate ppt. (Found: C, 60·6, 60·9; H, 7·0, 7·2; OMe, 6·3, 6·1%). This glucuronide was stable to boiling 0·5N-NaOH for 10 min.

*Acid hydrolysis of methyl* [*β*-(3-tert.-*butyl*-2-*hydroxy*-5-*methylphenyl*)-*ββ*-*dimethylethyl* 2:3:4-*tri*-O-*acetyl*-*β*-D-*glucopyranosid*]*uronate*. The crystalline glucuronide (280 mg.) in methanol (50 ml.) and 2N-HCl (25 ml.) was boiled under reflux for 3 hr. in a slow stream of $N_2$. On cooling, the mixture was extracted with light petroleum (b.p. 60–80°) (2 × 50 ml.). Removal of the solvent *in vacuo* gave long colourless crystalline plates (101 mg., 83%), m.p. 95–96°. Recrystallization from ethanol or sublimation *in vacuo* yielded colourless plates of *β*-(3-tert.-*butyl*-2-*hydroxy*-5-*methylphenyl*)-*ββ*-*dimethylethanol*, m.p. 110° (Found: C, 76·5, 76·9; H, 10·6, 10·5; OMe, nil. $C_{15}H_{24}O_2$ requires C, 76·2; H, 10·25%).

*Acid and enzymic hydrolysis of the crude ether glucuronide* (*compound C*). Ether-soluble glucuronide gum (fraction 1) (200 mg.) was applied to Whatman no. 17 paper and run with solvent system C (Table 2). The band of compound C was cut out and eluted with methanol. Removal of the methanol *in vacuo* yielded a slightly yellowish gum which failed to crystallize from numerous solvents. After hydrolysis of the gum (about 20 mg.) by both methanolic HCl and *β*-glucuronidase (as described above), followed by paper chromatography of an ether extract, a compound with the same $R_F$ in two solvent systems (A and B, Table 1) as *β*-(3-*tert.*-butyl-2-hydroxy-5-methylphenyl)-*ββ*-dimethylethanol was identified.

*Isolation of methyl* (3:5-*di*-tert.-*butyl*-4-*hydroxybenzoyl* 2:3:4-*tri*-O-*acetyl*-*β*-D-*glucopyranosid*)*uronate*. The mother liquors remaining from the lead and ether-soluble glucuronide preparations after trituration with absolute ethanol and separation of the acetylated-methylated glucuronide of compound C, should contain the corresponding derivative of glucuronide B. Purifications and fractional separations in numerous solvents failed to effect crystallization of this compound. 3:5-Di-*tert.*-butyl-4-hydroxybenzoic acid was isolated, however, after acid hydrolysis of this residual gum.

Ether-soluble glucuronide gum (fraction 1) (3·28 g.) from 670 ml. of fresh urine from rabbits given BHT (5 g.), was methylated with diazomethane and then acetylated in acetic anhydride (25 ml.) with perchloric acid (1 drop of aq. 60%) as described by Mead, Smith & Williams (1955). Trituration of the dried precipitated gum with absolute ethanol formed long colourless plates (0·84 g.) of methyl [*β*-(3-*tert.*-butyl-2-hydroxy-5-methylphenyl)-*ββ*-dimethylethyl 2:3:4-tri-*O*-acetyl-*β*-D-glucopyranosid]uronate, m.p. 169° (decomp.); its identity was confirmed by a mixed-m.p. determination and u.v.-absorption spectrum. The residual gum (2·0 g.) was extracted with light petroleum (b.p. 60–80°) to remove any free 3:5-di-*tert.*-butyl-4-hydroxybenzoic acid and after dissolving in methanol (40 ml.) was remethylated and reacetylated as described above. The dried gum, which contained microcrystals, m.p. 118–120° (micro-hot-stage), was dissolved in ethanol (10 ml.) and added to a column of acid-washed alumina. Three equal fractions were collected on elution with ethanol–acetic acid (1:1, v/v). The first fraction, after removal of the solvent *in vacuo*, yielded a slightly coloured amorphous mass (0·38 g.), which on trituration with absolute ethanol gave a mixture of colourless crystals and a glass-like gum. The crystals were separated and quickly washed with ethanol (42 mg., m.p. 130–131°). Recrystallization from ethanol yielded fine colourless needle-like plates of *methyl* (3:5-*di*-tert.-*butyl*-4-*hydroxybenzoyl* 2:3:4-*tri*-O-*acetyl*-*β*-D-*glucopyranosid*)*uronate*, m.p. 136–137° (decomp.), $[\alpha]_D^{22}$ −35·0° in methanol (*c*, 1·0); light-absorption max., 217 and 267·5 mμ (ε 24 200 and 17 970 respectively) in 95% ethanol [Found: C, 59·8; H, 6·9; OMe, 5·8; $CH_3$·CO, 22·8 (corrected value: a blank determination with 3:5-di-*tert.*-butyl-4-hydroxybenzoic acid gave an acetyl value of 6·4). $C_{28}H_{38}O_{12}$ requires C, 59·4; H, 6·8; OMe, 5·5; $3CH_3$·CO, 22·8%]. A mixed-m.p. determination with the acetylated-methylated glucuronide from the urine of rabbits given 3:5-di-*tert.*-butyl-4-hydroxybenzoic acid was not depressed, but a mixed m.p. with the acetylated-methylated glucuronide of compound C (m.p. 167°) was lowered to 121–125°.

*Acid hydrolysis of methyl* (3:5-*di*-tert.-*butyl*-4-*hydroxybenzoyl* 2:3:4-*tri*-O-*acetyl*-*β*-D-*glucopyranosid*)*uronate*. The crystalline glucuronide (100 mg.) in methanol (25 ml.) and 2N-HCl (25 ml.) were boiled under reflux for 4 hr. in a slow stream of $N_2$. On cooling, water (25 ml.) was added and the mixture extracted with peroxide-free ether (3 × 50 ml.). Removal of the ether *in vacuo* after drying over anhydrous $MgSO_4$ gave yellowish crystals (38 mg., 66·4%), m.p. 185–187°. Paper chromatography with solvent system A (Table 1) showed the presence of 3:5-di-*tert.*-butyl-4-hydroxybenzoic acid and a trace of methyl 3:5-di-*tert.*-butyl-4-hydroxybenzoate. Vacuum sublimation of the crystals gave pure 3:5-di-*tert.*-butyl-4-hydroxybenzoic acid, m.p. 206–207° which was not depressed on admixture with the authentic acid.

*Acid and enzymic hydrolysis of the crude ester glucuronide* (*compound B*). Ether-soluble glucuronide gum (fraction 1) (200 mg.) was applied to Whatman no. 17 paper and run with solvent system C (Table 2). The band of compound B was separated and eluted with methanol. Removal of the methanol *in vacuo* yielded a slightly yellow gum which failed to crystallize from numerous solvents, even after rechromatography. After hydrolysis of the gum (about 20 mg.) by both methanolic HCl and *β*-glucuronidase, followed by paper chromatography of an ether extract and

of the residual mother-liquor concentrates, the only compound identified was 3:5-di-*tert.*-butyl-4-hydroxybenzoic acid.

*Acid hydrolysis of the glycine conjugate (compound D).* Ether-soluble gum (fraction 2) (40 mg.) was applied to paper and developed with solvent system C (Table 2). The band of the glycine conjugate was cut out and eluted with water (10 ml.). After hydrolysis with HCl, both glycine and 3:5-di-*tert.*-butyl-4-hydroxybenzoic acid were identified by chromatographic analysis.

### *Metabolites of 3:5-di-*tert.*-butyl-4-hydroxybenzoic acid*

*Isolation of unconjugated 3:5-di-*tert.*-butyl-4-hydroxybenzoic acid.* Urine (400 ml., pH 5·85) from a rabbit given 3:5-di-*tert.*-butyl-4-hydroxybenzoic acid (1·95 g.) in three consecutive daily doses, was extracted by shaking with peroxide-free ether (2 × 400 ml.). The extract was dried over anhydrous $MgSO_4$ and the solvent taken off *in vacuo* to yield a slightly yellow crystalline product. Vacuum sublimation (100–110°, 0·05 mm. Hg) gave colourless crystalline plates (38 mg., 2·0% of the dose), m.p. 210° which was undepressed on admixture with authentic 3:5-di-*tert.*-butyl 4-hydroxybenzoic acid. No other compound was detected in the ether extract by paper chromatography in solvent system A (Table 1).

*Isolation of methyl (3:5-di-*tert.*-butyl-4-hydroxybenzoyl 2:3:4-tri-O-acetyl-β-D-glucopyranosid)uronate.* Urine (400 ml.) after removal of the non-conjugated phenols (see above) was adjusted to pH 2·2 with dil. HCl and extracted by shaking with peroxide-free ether (3 × 400 ml.). The extract was dried over anhydrous $MgSO_4$ and the solvent removed *in vacuo* to yield a white friable powder (2·75 g., 53·9%). Chromatographic examination of this material with solvent systems C, E and F (Table 2) gave two spots with $R_F$ values and colour reactions identical with those of compounds B and D (Table 2). No other glucuronide-reacting compounds nor any other metabolites were detected in the residual urine, which gave negative Fehling and Benedict tests. The ether-soluble powder (1·6 g.) was methylated with diazomethane and acetylated with perchloric acid as described above. The precipitated gum was washed, dried and then triturated with absolute ethanol. The completely crystalline product (1·8 g.), m.p. 127–128°, was repeatedly recrystallized from ethanol to yield long colourless needles or prisms of methyl (3:5-di-*tert.*-butyl-4-hydroxybenzoyl-2:3:4-tri-*O*-acetyl-β-D-glucopyranosid)-uronate, m.p. 137·5–138° (decomp.) undepressed on admixture with the methylated–acetylated glucuronide of compound B from BHT urine, $[\alpha]_D^{20}$ −35·0° in methanol (*c*, 1·0) (Found: C, 59·4, 59·9; H, 6·8, 7·0; OMe, 6·3%).

*Acid hydrolysis of the glycine conjugate.* The ether-soluble powder (150 mg.) was applied to paper and run with solvent system C (Table 2). The band of the glycine conjugate was cut out and eluted with water (15 ml.). After hydrolysis, both glycine and 3:5-di-*tert.*-butyl-4-hydroxybenzoic acid were identified.

## RESULTS

*Excretion of normal metabolites.* The mean daily excretions (± S.D.), by undosed rabbits were: free phenols, 0·544 ± 0·135; total phenols, 0·607 ± 0·177; total glucuronides, 0·366 ± 0·184; ester glucuronides, 0·230 ± 0·058; ethereal sulphates, 0·164 ± 0·080; glycine conjugates, 0·323 ± 0·103 m-mole.

### *Metabolites of butylated hydroxytoluene*

The results of the analytical determinations of metabolites excreted in rabbit urine after the administration of single and repeated daily doses of butylated hydroxytoluene are set out in Table 3. Excretion of all the metabolites is complete only after 3–4 days after dosing of the animals. About 54% of the administered dose is accounted for, and the recovery of metabolites after four and five repeated doses is not very different from that of single doses of butylated hydroxytoluene.

All the metabolites of butylated hydroxytoluene as indicated by the initial analyses, with the exception of the ethereal sulphates, were satisfactorily accounted for by the paper-chromatographic examination of urine. Methods for the location and identification on paper chromatograms of the ethereal sulphates were not successful.

*Non-conjugated phenols.* When urines were ether-extracted at pH 5·5–6·5, only one compound was present in the extract. This was shown to be identical with 3:5-di-*tert.*-butyl-4-hydroxybenzoic acid. No unequivocal evidence for butylated hydroxytoluene by chromatographic examinations of solvent extracts or steam-distillates of urine was found and if any is excreted the amount is likely to be very small.

*Glucuronide conjugates.* The excretion of glucuronides accounts for almost 36% of doses of butylated hydroxytoluene (Table 3) and 16% could be attributed to an ester-type glucuronide (compound B). Paper-chromatographic analysis also confirmed the excretion of two different glucuronides (Table 2). These two metabolites were isolated from urine as the crystalline methylated–acetylated derivatives. The ester glucuronide (compound B) was shown to be identical by m.p., mixed m.p., chromatographic behaviour and u.v. spectrum with the fully acetylated-methylated glucuronide isolated from the urine of rabbits given 3:5-di-*tert.*-butyl-4-hydroxybenzoic acid (see below). The compound is thus formulated as 3:5-di-*tert.*-butyl-4-hydroxybenzoyl β-D-glucosiduronic acid. Elementary analysis of the acetylated–methylated ether glucuronide (compound C) and its crystalline aglycone indicated oxidation of the butylated hydroxytoluene molecule with one atom only of oxygen. The i.-r. spectrum of both the aglycone and glucuronide showed the presence of strong bands at 2·90 μ (the sterically hindered hydroxyl group), at 9·45 μ (a primary alcohol side chain) and at 12·00 μ (a phenolic *C*-methyl group). The spectra support the contention that oxidation of a *tert.*-butyl group to a (1-hydroxy-2:2-dimethyl)ethyl group has taken place. On this evidence the aglycone is

Table 3. *Metabolites excreted in rabbit urine after the administration of single or repeated daily doses of butylated hydroxytoluene*

Results are expressed as a percentage of the dose (single animals). Free phenols: ester glucuronide + non-conjugated phenols. Values obtained by difference are in parentheses.

| No. of doses | Daily dose (m-moles/kg.) | Non-conjugated phenols | Free phenols | Total phenols | Total glucuronides | Ester glucuronide | Ether glucuronide | Ethereal sulphate | Glycine conjugate |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 2·28 | — | 26·2 | 31·7 | 35·5 | — | — | 5·3 | — |
| 1 | 2·43 | — | 20·4 | 24·4 | 38·7 | — | (17·9) | 3·0 | — |
| 4 | 3·64 | 4·1 | 19·0 | 22·0 | 35·4 | 18·4 | — | 3·0 | — |
| 4 | 1·82 | 9·0* | — | — | — | — | — | — | — |
| 5 | 3·65 | — | — | — | 33·2 | 14·0 | (18·2) | — | — |
| 6 | 3·63 | — | 21·2 | — | 36·1 | 16·0 | (20·1) | — | — |
| 5 | 3·64 | 8·0 | — | — | — | — | — | 7·6 | — |
| | 3·64 | — | 30·9 | 30·7 | 36·6 | 14·5 | (22·1) | 10·3 | 1·3 |
| | Mean | 8·4 | 24·1 | 27·2 | 35·9 | 16·0 | (19·6) | 6·0 | 1·3 |
| | | | | | | | | 8·0 | 1·3 |

* Mean of four separate experiments where only the non-conjugated phenols were determined.

identified as β-(3-*tert.*-butyl-2-hydroxy-5-methylphenyl)-ββ-dimethylethanol and the glucuronide is formulated as β-(3-*tert.*-butyl-2-hydroxy-5-methylphenyl)-ββ-dimethylethyl β-D-glucosiduronic acid. The aglycone has not yet been synthesized.

*Glycine conjugate.* This metabolite (compound D) was shown to be chromatographically identical with the glycine conjugate that is present in urine after the administration of 3:5-di-*tert.*-butyl-4-hydroxybenzoic acid to a rabbit. Hydrolysis of the conjugate eluted from paper chromatograms confirmed that its structure is 3:5-di-*tert.*-butyl-4-hydroxyhippuric acid.

*Ethereal sulphates.* About 8 % (± 1·9) of doses of butylated hydroxytoluene were excreted as ethereal sulphates (see Table 3).

*Mercapturic acids.* No consistent indication of the excretion of butylated hydroxytoluene in the form of mercapturic acid conjugates was found.

### *Metabolites of 3:5-di-tert.-butyl-4-hydroxybenzoic acid*

After the oral administration of 3:5-di-*tert.*-butyl-4-hydroxybenzoic acid in three consecutive daily doses to a rabbit, more than 80 % was accounted for as ester-type glucuronide and about 9 % as a glycine conjugate. Excretion of metabolites was normal on the fifth day after dosing commenced. The recovery of the metabolites is set out in Table 4. The glucuronide was 3:5-di-*tert.*-butyl-4-hydroxybenzoyl β-D-glucosiduronic acid and the glycine conjugate was 3:5-di-*tert.*-butyl-4-hydroxyhippuric acid.

Table 4. *Metabolites excreted in rabbit urine after the administration of 3:5-di-tert.-butyl-4-hydroxybenzoic acid daily over three consecutive days*

Results are expressed as a percentage of the dose (single animals).

| | Total dose (m-moles/kg.) | |
|---|---|---|
| Non-conjugated phenols | 2·25 | 4·70 |
| Free phenols (non-conjugated phenol + ester glucuronide) | 5·2 | 2·0 |
| Total phenols | 90·5 | — |
| Total glucuronides | 96·2 | — |
| Ester-type glucuronide | 84·0 | 87·5 |
| Glycine conjugate | 83·5 | — |
| | 8·8 | — |

## DISCUSSION

The usual methods employed for the estimation of phenols in biological materials (Bray & Thorpe, 1954) cannot be used for butylated hydroxytoluene because of the sterically hindered hydroxyl group. Although the compound reacts with ferric chloride-

2:2'-dipyridyl (Anglin, Mahon & Chapman, 1956; Eastman Chemical Products Inc., 1957), in our hands the method was not sufficiently accurate nor applicable to the routine analysis of butylated hydroxytoluene in urine samples. No free butylated hydroxytoluene was found by the ferric chloride-2:2'-dipyridyl method, nor was it seen in any of the paper chromatograms. Similarly, there was no evidence of conjugates hydrolysable to butylated hydroxytoluene itself. Furthermore, no evidence was found for the conjugation of the metabolites of butylated hydroxytoluene in the normal manner through the hindered hydroxyl group. Confirmation of a free unconjugated hydroxyl group was obtained from measurements of the infrared spectra of these metabolites (cf. Bohn & Campbell, 1957).

From the metabolites isolated it is clear that butylated hydroxytoluene is oxidized either by conversion of the methyl group in position 1 into a carboxyl group or by ω-oxidation of one of the *tert.*-butyl groups to the primary alcohol stage, and that the conjugates are formed through one of these two new groups. As the phenolic hydroxyl of butylated hydroxytoluene is sterically hindered, the pathway by ω-oxidation is probably the one followed for ethereal sulphate synthesis. The conjugation of certain types of primary alcohols with sulphuric acid is known to occur (Williams, 1959). Both types of oxidation have been reported previously: about 10% of an oral dose of *p*-cresol is excreted as free and conjugated *p*-hydroxybenzoic acid (Bray, Thorpe & White, 1950) and up to 90% of a dose of *tert.*-butylbenzene is oxidized by rabbits to 2:2-dimethyl-2-phenylethanol and excreted as a glucuronide (Robinson & Williams, 1955). As far as we are aware, this is the first example of a single compound following both oxidative routes and involving three different conjugating agents.

The study has shown that rabbits can cope with relatively large single doses of butylated hydroxytoluene (Table 3) and up to three daily doses of 3:5-di-*tert.*-butyl-4-hydroxybenzoic acid (Table 4). That four and five consecutive daily doses of butylated hydroxytoluene have cumulative and lethal effects is, however, not very reassuring. The metabolites described account for 54·1% (±7·2%) of the dose. The fate of the remainder is unknown.

## SUMMARY

1. The metabolism of the antioxidant 3:5-di-*tert.*-butyl-4-hydroxytoluene (BHT) and its oxidation product, 3:5-di-*tert.*-butyl-4-hydroxybenzoic acid, has been studied in the rabbit.

2. Rabbits excreted BHT mainly as glucuronides, some 16·0% as an ester glucuronide and 19·4% as an ether glucuronide. Unconjugated phenol (8·4%), ethereal sulphate (8·0%) and a glycine conjugate (1·8%) were also excreted. The distribution and recovery of metabolites when four and five daily doses were given was not significantly different from that of single doses of BHT.

3. After three consecutive daily doses of 3:5-di-*tert.*-butyl-4-hydroxybenzoic acid, a rabbit excreted 84·0% of the total dose as ester glucuronide, 8·8% as a glycine conjugate and 5·2% was not conjugated.

4. The ester glucuronide, isolated from the urine after both BHT and the acid as the triacetyl methyl ester, is 3:5-di-*tert.*-butyl-4-hydroxybenzoyl β-D-glucosiduronic acid.

5. The ether glucuronide, isolated from BHT urine as the triacetyl methyl ester, is β-(3-*tert.*-butyl-2-hydroxy-5-methylphenyl)-ββ-dimethylethyl β-D-glucosiduronic acid.

6. The glycine conjugate of both BHT and the acid is 3:5-di-*tert.*-butyl-4-hydroxyhippuric acid.

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# The Absorption and Excretion of Butylated Hydroxytoluene (BHT) in the Rat

J. W. DANIEL and J. C. GAGE

*Imperial Chemical Industries Limited, Industrial Hygiene Research Laboratories, Alderley Park, Cheshire, England*

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Abstract—The concentration of butylated hydroxytoluene (BHT; 3,5-di-*tert*-butyl-4-hydroxytoluene) in the fat and liver of rats fed on diets containing 0·5 and 1·0% BHT for up to 50 days has been determined by a colorimetric method. In both tissues the concentration rapidly reached a fluctuating plateau; with the 0·5% diet this averaged around 30 ppm for males and 45 ppm for females in fat, while in the liver the values were much lower. When these rats were returned to normal diet the half-life of BHT in fat was found to be 7–10 days.

After a single oral dose of [$^{14}$C]BHT, 80–90% of the administered radioactivity was excreted within 4 days; in females about 60% of the dose was found in the urine and in males about 25%. Two and 4 days after dosing, the amounts retained in the body were in the region of 15 and 4% respectively, the bulk of this being in the gut. As an appreciable proportion of the dose was excreted in bile, it is probable that the excretion of BHT metabolites is delayed by an enterohepatic circulation.

When a single dose of [$^{14}$C]BHT was administered to rats fed on a diet containing 0·5% BHT, the excretion was somewhat more rapid, and about 40% of the dose appeared in the urine in both males and females.

## INTRODUCTION

The fate of the antioxidant, butylated hydroxytoluene (BHT; 3,5-di-*tert*-butyl-4-hydroxytoluene), after administration to experimental animals has been studied by Dacre (1961), by Holder, Ryan & Wright (1962) and by Ladomery, Ryan & Wright (1963). Dacre (1961) found that a rabbit dosed orally with BHT excreted about 54% in the urine over a period of 3 days; none of this was uncharged BHT and the bulk appeared to be present as glucuronide conjugates after oxidation of BHT at the methyl group or at one of the *tert*-butyl groups. Holder *et al.* (1962) studied the excretion of radioactivity in urine after an intraperitoneal injection of tritium-labelled BHT into the rat, and Ladomery, Ryan & Wright (1963) extended this investigation by the administration of $^{14}$C-labelled BHT. These authors found that the amount of radioactivity in the urine corresponded to about 33% of the dose over a period of 4 days and they reported no radioactivity in the faeces.

These experiments suggested that there might be an appreciable storage of BHT in the faeces after a single dose. In order to test this inference, 2 series of experiments have been undertaken: in the first, the concentration of BHT in body fat and liver has been studied during and after administration to rats of a diet containing BHT; and in the second case, the excretion of radioactivity in urine and faeces and the residual radioactivity in faeces has been studied after oral administration of a single dose of [$^{14}$C]BHT.

## EXPERIMENTAL

*Materials.* The sample of BHT used was the "Topanol" grade supplied by Imperial Chemical Industries Limited (Alderley Park, Cheshire); it contained not less than 99·5% of

3,5-di-*tert*-butyl-4-hydroxytoluene. Radioactive BHT was obtained from the New England Nuclear Corporation (Boston, Mass.); this was labelled with $^{14}C$ in the *tert*-butyl group with a specific activity of 2·4 mc/g. The presence of only one radioactive substance was indicated by thin-layer chromatography.

*Animals and dosage.* The animals used were Alderley Park strain SPF albino Wistar rats with an average body weight of 225-275 g unless otherwise stated. Oral doses of BHT solutions were administered by stomach tube, and the rats were housed in individual metabolism cages (Gage, 1961), for the separate collection of urine and faeces.

Solutions of BHT for oral administration to rats were prepared by warming the samples in olive oil. The radioactivity of the solution administered was checked by diluting a portion with dioxan and following the procedure described below for urine.

The diets used for the experiments reported in Table 1 and Figs. 1 and 2 were prepared by dissolving the required amount of BHT in 100 ml maize oil and adding this to 0·9 kg maize extract. Approximately 750 ml water were added in successive instalments with constant stirring and the whole was mixed with 4 kg powdered rat diet. After extrusion of the maize diet from a sausage-meat machine, the diet was dried in a vacuum oven. In the experiment reported in Table 4 the required amount of BHT was mixed into the dry powdered rat diet which was then damped down with a known amount of water for presentation to the rats.

*Colorimetric determination of BHT.* The most sensitive colorimetric method for the determination of trace amounts of BHT is based on a coupling reaction with diazotized di-*o*-anisidine (Szalkowski & Garber, 1962). This method is not specific as other phenols may give a positive reaction, but BHT may be separated from most interfering substances by virtue of its volatility in steam. A simple steam distillation of BHT is a very slow process and Anglin, Mahon & Chapman (1956) have described a method in which superheated steam is blown into an aqueous solution of BHT containing calcium chloride to raise the boiling point. Tests of this method in this laboratory have shown a poor recovery of known amounts of BHT, probably due to partial destruction of the compound at the elevated temperature. Sloman, Romagnoli & Cavagnol (1962) passed steam into an aqueous solution of BHT containing isopropanol, in order to facilitate the distillation of the BHT at the lower temperature. When their method was tested in this laboratory it was found that the isopropanol in the distillate seriously interfered with the chloroform extraction of the colour produced when BHT was coupled with diazotized di-*o*-anisidine. It was assumed that the failure to obtain satisfactory results by this method was due to the quality of the isopropanol used, but a fractional distillation of the solvent did not make the method satisfactory.

Methanol was found experimentally to be a more satisfactory solvent than isopropanol in the distillation of BHT. Virtually complete recoveries were obtained when steam was blown through aqueous methanol containing known amounts of BHT, and methanol was found not to interfere in the subsequent extraction of the coloured azo compound with chloroform. Less satisfactory recoveries were obtained when known amounts of BHT were added to a methanol extract of fat which was then diluted with water and steam distilled. As it seemed likely that the BHT was being retained in the methanol-soluble fat which separated in the presence of water, the methanol extract of fat containing BHT was chilled to −30°C and the clear solution decanted from the separated fatty material. Steam distillation of the methanol extract then yielded recoveries approaching 100%. Methanol was found to be not entirely a satisfactory solvent for the extraction of BHT from fat, so the extraction of anhydrous fat was made with ether, and after careful evaporation of the ether the residue was dissolved in methanol which was subjected to steam distillation after chilling.

*Determination of BHT in fat and liver.* The following method was used for the determination of BHT in fat and liver. The sample was ground with sand and anhydrous sodium sulphate and extracted overnight with 80 ml ether in a Soxhlet apparatus. The ether was allowed to evaporate on a warm-water bath and the residue was shaken with 80 ml methanol for 5 min. The flask and its contents were chilled to −30°C for 30 min and the supernatant liquid was filtered through cotton wool into a 100 ml flask; the flask was washed with chilled methanol and the washings added to the first extract and the total volume was then adjusted to 100 ml with methanol. The methanol solution was diluted with 50 ml water and subjected to steam distillation in the apparatus described by Sloman *et al.* (1962). A total of 200 ml distillate was collected, mixed and divided into 2 equal portions for the determination of BHT. Each 100 ml portion of distillate was placed in a 250 ml separating funnel and 20 ml p-anisidine hydrochloride reagent (Szalkowski & Gerber, 1962) and 8 ml of 0·3% (w/v) sodium nitrite solution were added. After mixing, the solution was left for 10 min and then shaken with 15 ml chloroform for 30 sec. The chloroform was transferred to a measuring cylinder and the volume adjusted to 12 ml with methanol. The optical density was measured immediately in 4-cm cells at 520 mμ. As the colour was unstable and sensitive to light, determinations were made singly and the separating funnels and measuring cylinders were covered with metal foil. A calibration graph was constructed by subjecting known amounts of BHT in 100 ml of 50% (v/v) aq methanol to the same analytical procedure. The graph was linear with a slope of 0·0253 optical density unit/μg BHT.

In order to test the recovery of BHT from fat and liver, measured volumes of a solution of BHT were injected by a micrometer syringe into fat and liver taken from normal rats. The tissues were then subjected to the analytical procedure described above; recoveries of 95% or better were obtained when 4 and 16 ppm BHT were added to 5 g fat, and 3 ppm were added to 7 g liver.

*Measurement of radioactivity in urine and faeces.* Urine samples with an activity of more than about 30 dps/ml were diluted (1:10) with water and 1 ml of the dilution was added to 15 ml scintillator solution (50·2 g naphthalene, 5·0 g PPO, 0·125 g POPOP in 500 ml dioxan). With less active samples, 1 ml water and 1 ml urine were measured into vials and 15 ml of the above scintillator solution containing 4% (w/v) colloidal silica (low in iron) (British Drug Houses Ltd., Poole, Dorset) was added. The vial was capped and shaken to induce gelling.

In the experiments reported in Tables 2 and 3, the daily output of faeces was weighed and a 10% (w/v) homogenate in water was prepared. A 2-ml portion of this homogenate was transferred to a flask fitted with a dropping funnel and an air entry and exit tube, the latter leading to a bubbler containing water and then to two spiral absorbers in series each containing 6 ml N-sodium hydroxide solution. Air free from carbon dioxide was drawn through the system and 10 ml Van Slyke acid mixture (Van Slyke & Folch, 1940) was added to the flask through the dropping funnel. The temperature of the flask was slowly brought up to 90°C and maintained at that temperature for 45 min. A 2-ml portion from each absorber was transferred to a glass vial and 13 ml of the scintillator solution mentioned above, containing 4% colloidal silica, was added. Owing to difficulties in obtaining reproducible samples of the faecal homogenate the procedure was modified for the experiment described in Table 4. Faeces were homogenized in acetone, the acetone insoluble material was separated, washed with acetone, dried and mixed. A sample of the combined acetone extracts was added to scintillator solution and counted, and a sample of the acetone insoluble powder was acid-oxidised and counted as sodium carbonate as above. As there was some evidence

that the acid-oxidising mixture did not completely destroy the acetone-insoluble material, and as total recovery of radioactivity in these experiments was somewhat low, the method was further modified by burning a sample of the acetone-insoluble powder in a stream of carbon dioxide free air in a furnace (Peets, Florini & Buyske, 1960), and absorbing the carbon dioxide produced in bubblers containing 10 ml of 5 N-sodium hydroxide solution. A portion of the absorbing solution was titrated to determine the extent of neutralization, and then the calculated amount of carbon dioxide was passed through the solution to neutralize the free sodium hydroxide. Duplicate 3-ml portions of the solution were added to 17 ml scintillator solution containing 4% silica. This method was used for the experiments reported in Table 5. Samples of tissue were prepared for counting by oxidation in a furnace in the manner described above for the acetone insoluble faecal powder.

In all cases duplicate determinations were made and a further 2 duplicate samples were taken for the determination of counting efficiency; for the direct counting of urine and the acetone extract of faeces these were fortified with [$^{14}$C]BHT or [$^{14}$C]hexadecane, while the sodium carbonate solutions obtained by acid oxidation or combustion were fortified with [$^{14}$C] sodium carbonate. The vials were counted for 5- or 10-min periods in a Packard Model 3214 Automatic Tri-Carb Spectrometer.

## RESULTS

*The tissue retention of BHT during continuous feeding*

A diet containing 1% BHT was fed to a group of 5 male and 5 female rats for 50 days. One of each sex was killed on days 22 and 50 of feeding, and a sample of abdominal fat was removed for analysis. The survivors were then returned to normal diet for 18 days after which they were killed and samples of body fat taken for analysis. The results of this experiment are shown in Table 1.

Table 1. *BHT content of fat of rats receiving diets containing 1% BHT for up to 50 days*

| Duration of feeding (days) | BHT content of fat (ppm) | |
|---|---|---|
| | Male | Female |
| 0 | — | — |
| 22* | 53 | 26 |
| 50* | 49 | 34 |
| 50† | 3·0 | 4·2 |

*1 rat of each sex was killed on this day for fat analysis.
†3 rats of each sex were returned to normal diets for 18 days prior to fat analysis.

As the results in Table 1 indicated that the concentration of BHT in fat had reached a steady state within 3 wk of continuous feeding, the experiment was repeated, this time with a diet containing 0·5% BHT, and with a group of male rats (average body weight 380 g), and a group of female rats (average body weight 240 g). Control groups were also maintained as before on a normal diet. Rats were killed at intervals from the experimental and control groups during the 35-day feeding period, and liver and body fat were taken for analysis. After 35 days on the diet the survivors were transferred to a normal diet and rats were taken at intervals for fat analysis over a further 25-day period. The results of these analyses are shown graphically in Fig. 1 and 2.

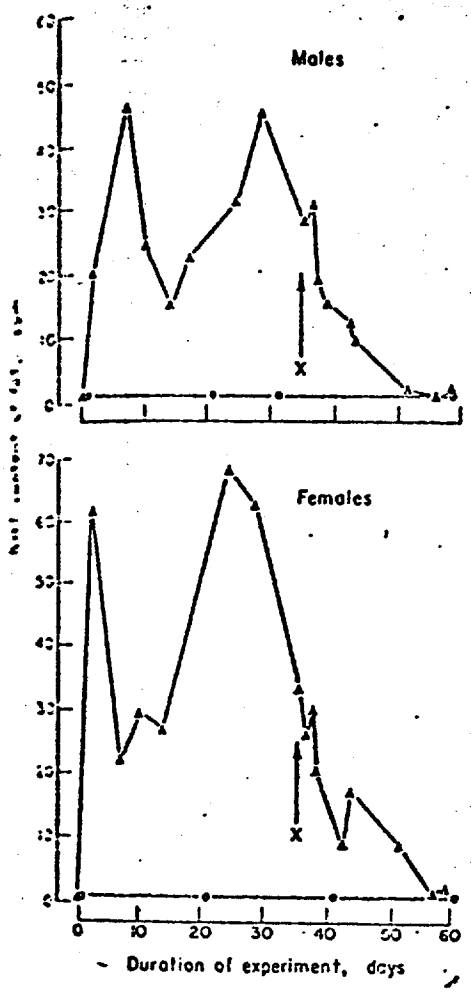


FIG. 1. Storage of BHT in fat of rats given 0·5% BHT in the diet (▲) for 35 days followed by return to normal diet. Results of animals receiving normal diet throughout are indicated by ○. X—start of return to normal diet.

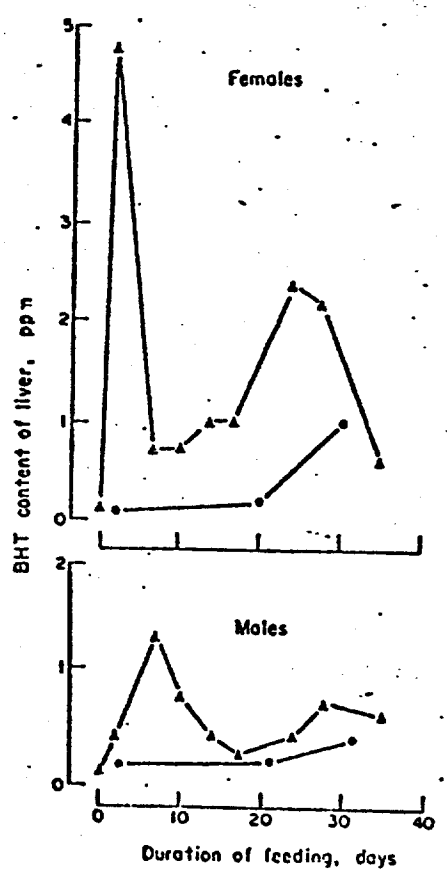


FIG. 2. Storage of BHT in liver of rats given 0·5% BHT in the diet (▲) for 35 days. Results of animals receiving normal diet throughout are indicated by ○.

*Excretion of radioactivity after single oral doses of [$^{14}$C]BHT*

The radioactivity excreted in urine and faeces of rats receiving [$^{14}$C]BHT in single doses of 1 or 2·4 mg/rat is shown in Tables 2 and 3. This experiment was then repeated in rats before and after the administration of a diet containing 0·5% BHT (unlabelled). A dietary concentration is equivalent to a daily oral dose of approximately 100 mg/rat. An initial dose of 7·8 μc [$^{14}$C]BHT was administered together with 100 mg unlabelled BHT. On the day after this initial dose, the group of rats received the 0·5% BHT diet and the urine and faeces were collected for 7 days. The administration of the BHT diet continued for a total of 21 days; the rats were then transferred to a normal diet and immediately dosed with 7·8 μc [$^{14}$C]BHT together with 100 mg unlabelled BHT. Urine and faeces were collected during a period of 4 days after this second radioactive dose. Radioactivity measurements on the excreta are shown in Table 4.

**Table 2.** *Radioactivity excreted in urine and faeces of rats given single oral doses of 1 mg (2·4 μc) [$^{14}$C]BHT*

| | Percentage of dose excreted daily in | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Urine at day | | | | | | | Faeces* at day | | | | |
| | | | | | | 1–7 | | | | | | Percentage of dose excreted |
| Sex | 1 | 2 | 3 | 4 | 5–7 | Total | average | 1 | 2 | 3 | 4 | 1–4 | |
| Male | 5·8 | 5·1 | 2·7 | 2·4 | 0·6 | 16·6 | | | | | | | |
| Male | 6·7 | 8·3 | 3·8 | 1·4 | 0·6 | 20·8 | 24·0 | 5·2 | 20·2 | 11·7 | 5·0 | 42·1 | 66·1 |
| Male | 6·3 | 11·6 | 6·6 | 3·0 | 0·6 | 28·1 | | | | | | | |
| Male | 6·7 | 12·4 | 8·3 | 2·4 | 0·6 | 30·4 | | | | | | | |
| Female | 19·8 | 13·7 | 5·6 | 2·4 | 0·3 | 41·8 | | | | | | | |
| Female | 28·6 | 15·8 | 9·3 | 3·4 | 0·3 | 57·4 | 37·2 | 7·9 | 13·5 | 11·5 | 2·0 | 34·9 | 72·1 |
| Female | 4·8 | 5·0 | 1·7 | 0·6 | 0·3 | 12·4 | | | | | | | |

*Results are for pooled rats of either sex.

**Table 3.** *Radioactivity excreted in urine and faeces of rats given single oral doses of 2·4 mg (5·8 μc) [$^{14}$C]BHT*

| | | Daily excretion expressed as percentage of dose at day | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Sex | Route of excretion | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 1–8 | Percentage of dose excreted |
| Male | Urine | 7·3 | 10·5 | 2·0 | 1·4 | 2·1 | 0·34 | 0·32 | 0·05 | 24·0 | 67·6 |
| | Faeces | 10·5 | 18·0 | 8·5 | 4·1 | 1·6 | 0·7 | 0·06 | 0·16 | 43·6 | |
| Male | Urine | 6·2 | 11·5 | 6·4 | 2·6 | 1·1 | 0·46 | 0·82 | 0·21 | 29·3 | 72·8 |
| | Faeces | 7·1 | 21·2 | 10·0 | 2·7 | 1·7 | 0·65 | 0·03 | 0·09 | 43·5 | |
| Female | Urine | 16·3 | 18·2 | 7·4 | 2·9 | 1·3 | 0·58 | 0·57 | 0·28 | 47·5 | 79·7 |
| | Faeces | 0·0 | 19·6 | 6·2 | 3·45 | 1·47 | 1·37 | 0·05 | 0·04 | 32·2 | |
| Female | Urine | 17·2 | 17·8 | 3·6 | 2·8 | 1·0 | 0·54 | 0·26 | 0·08 | 43·3 | 64·5 |
| | Faeces | 0·0 | 15·1 | 0·0 | 3·1 | 2·2 | 0·7 | 0·05 | 0·02 | 21·2 | |

Table 4. *Excretion of $^{14}C$ in urine and faeces of rats given single oral doses of 103 mg (7·8 μc) [$^{14}C$]BHT before and after feeding on 0·5% BHT for 21 days*

| Sex and animal no. | Percentage of dose excreted daily in: Urine at day 1 | 2 | 3 | 4–6 | 7 | 1–7 Total | 1–7 Average | Faeces at day 1 | 2 | 3 | 4–6 | 7 | 1–7 | Percentage of dose excreted |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **During first 7 days on 0·5% BHT diet** | | | | | | | | | | | | | | |
| M1 | 4·0 | 19·4 | 9·4 | 4·0 | 0·3 | 37·1 | 29·3 | 3·4 | 11·5 | 14·6 | 12·5 | 0·0 | 42·0* | 71·3 |
| M2 | 6·0 | 8·3 | 3·0 | 4·0 | 0·15 | 21·5 | | | | | | | | |
| F1 | 10·0 | 18·2 | 4·9 | 2·3 | 0·1 | 35·5 | 31·5 | 0 | 25·3 | 9·4 | 8·2 | 0·0 | 42·9* | 74·4 |
| F2 | 5·6 | 13·5 | 5·9 | 2·3 | 0·2 | 27·5 | | | | | | | | |
| **On normal diet after 21 days on 0·5% BHT diet** | | | | | | | | | | | | | | |
| | 1 | 2 | 3 | 4 | | 1–4 Total | 1–4 Average | 1 | 2 | 3 | 4 | | 1–4 | |
| M1 | 20·6 | 11·9 | 3·1 | 0·3 | | 35·9 | | 4·5 | 25·5 | 6·8 | 3·2 | | 40·0 | 75·9 |
| M2 | 27·8 | 14·0 | 3·2 | 1·0 | | 46·0 | | 1·0 | 21·5 | 2·2 | 3·7 | | 28·4 | 74·4 |
| F1 | 24·5 | 30·4 | 5·4 | 0·9 | | 61·2 | 40·6 | 0·6 | 23·5 | 7·1 | 0·5 | | 31·7* | 72·3 |
| F2 | 5·5 | 11·5 | 2·3 | 0·7 | | 20·0 | | | | | | | | |

M=Male F=Female.
*Results are for pooled rats.

In Tables 2–5 it can be seen that the total recovery of radioactivity from the [illegible] amounted to approximately 75% of the dose administered. As there was evidence that oxidising acids used to treat faeces were not completely destroying the organic matter, a further experiment was undertaken in which faeces were extracted with acetone and the insoluble fraction was incinerated in a combustion furnace to yield $^{14}CO_2$. The results of several experiments using this method are given in Table 5 and show a more satisfactory recovery of radioactivity.

Table 5. *Excretion of $^{14}C$ by rats dosed orally with [$^{14}C$]BHT*

| No. of rats | Sex | Average body weight (g) | Dose (mg/rat) | Urine | Percentage of dose excreted by day 4 in Faeces: Acetone extract | Acetone insoluble | Percentage of dose excreted |
|---|---|---|---|---|---|---|---|
| 1 | Male | 170 | 1·16 | 10·7 | 58·1 | 21·7 | [illegible] |
| 5 | Female | 139 | 1·06 | 19·1 | 50·4 | 15·3 | [illegible] |
| 4 | Female | 150 | 103 | 58·5 | 24·8 | 3·4 | [illegible] |

*Retention of radioactivity in tissues*

The results of an experiment designed to demonstrate the retention of radioactivity in tissues after a single oral dose of 1·16 mg [$^{14}$C]BHT are shown in Table 6. Studies on the excretion of radioactivity in breath were also undertaken on these 2 rats. With the first rat the exhaled air was scrubbed first with toluene and then with sodium hydroxide solution (Gage, 1963), this operation being performed continuously for 48 hr. The amount of radioactivity extracted by the toluene and sodium hydroxide solution, expressed as a percentage

Table 6. *Retention of radioactivity in rat tissues 2 and 4 days after oral administration of 1·16 mg (2 μc) [$^{14}$C]BHT*

| Tissue | Percentage of dose retained in tissues after day 2: Per g | Total | 4: Per g | Total |
|---|---|---|---|---|
| Liver | 0·15 | 1·13 | 0·058 | [illegible] |
| Heart | 0·032 | 0·023 | 0·014 | [illegible] |
| Kidney | 0·03 | 0·034 | 0·001 | [illegible] |
| Lung | 0·035 | 0·039 | 0·015 | [illegible] |
| Spleen | 0·014 | 0·007 | 0·298 | [illegible] |
| Thymus | 0·017 | 0·007 | 0·002 | — |
| Brain | 0·0045 | 0·008 | 0·002 | [illegible] |
| Fat | 0·064 | 0·77* | 0·027 | [illegible]* |
| Blood | 0·05 | 0·85* | 0·017 | [illegible]* |
| Skin | 0·019 | 0·58* | 0·0056 | [illegible]* |
| Muscle | 0·009 | 0·67* | 0·0056 | 0·41* |
| Stomach | | | | [illegible] |
| Small intestine | | 11·25 | | [illegible]* |
| Caecum | | | | [illegible] |
| Large intestine | | | | [illegible] |
| Total .... | | 14·8 | | [illegible] |

*Estimated values (Donaldson, 1924).

of the dose, was 0·03 and 0·26 respectively. With the second rat, the exhaled air was passed through a pyrolysis tube and then scrubbed with sodium hydroxide solution; 0·14% of the dose was collected during the first 24 hr and 0·12% during the second 24 hr.

*Excretion of radioactivity in bile*

A polythene cannula (0·5 mm bore) was inserted into the bile duct of a male rat under [illegible] anaesthesia. An oral dose of 1·75 mg (4·2 μc) [$^{14}$C]BHT was administered to the rat which was then placed in a restraining cage. An unlimited supply of water containing [illegible] sodium chloride and 1% glucose was made available to the rat. Over a period of 40 hr after dosing, 20 ml bile was collected and this was found to contain 53% of the administered radioactivity. Bile was collected in a similar manner from a female rat which had been dosed with [$^{14}$C]BHT equivalent to 5·4 μc. In this rat 30 ml bile containing radioactivity, equivalent to [illegible]% of the dose administered, was collected over a period of 40 hr.

## DISCUSSION

The results in Tables 2-5 demonstrate that a single oral dose of [$^{14}$C]BHT is almost completely excreted by the rat within 4 days. As recoveries of radioactivity in the excreta and tissues approaching 90% were obtained when a satisfactory method for the oxidation of faeces was employed (Table 5), the lower recoveries in Tables 2, 3 and 4 must be attributed to an incomplete conversion of BHT metabolites in faeces to carbon dioxide by the acid oxidation method used, and it seems likely that the faecal excretion reported in these tables is low. There can be no doubt that the inadequate recoveries of BHT which have been reported by earlier investigators (Ladomery *et al.* 1963) is due to a failure to recognize the considerable faecal excretion.

The results reported in Tables 2 and 3 indicate that the overall excretion is slightly faster in the female than in the male rat, and that a larger proportion of the dose is excreted in the urine of females. This observation may be connected with the somewhat lower susceptibility of female rats to high doses of BHT which has been observed in this laboratory. The more rapid urinary excretion in females also occurred at the higher dose levels (Table 4). When a single dose was administered to rats fed on a diet containing 0·5% BHT for 21 days the excretion of radioactivity in urine is slightly more rapid and approaches 40% of the dose in both males and females; this may be related to the development of tolerance which has been observed in rats fed on diets containing BHT.

The measurement of residual radioactivity in tissues after a single oral dose of [$^{14}$C]BHT confirms the conclusions reached from excretion studies that there is little retention of BHT or its metabolites in rat tissues. In one experiment reported in Table 6, 14·8% of the dose was retained in the tissues 2 days after dosing and this had fallen to 3·8%, 4 days after dosing. The bulk of this residual radioactivity was located in the small intestine, even when the excretion of radioactivity in faeces had fallen to a low level. This observation, together with the findings of considerable excretion of radioactivity in bile and the somewhat higher concentration in liver than in other organs, provide strong evidence that a large proportion of the BHT metabolites are excreted in bile and then re-absorbed from the gut. As the somewhat low concentrations measured in other tissues decrease at about the same rate as does the amount in the gut, it seems likely that it is enterohepatic circulation, and not tissue retention, which is responsible for the kinetics of the excretion of BHT metabolites.

The low retention of BHT in the tissues is also demonstrated by the chemical analysis of

fat and liver tissues from rats receiving BHT in the diet. Fig. 1 indicates that the content of fat rapidly attains a somewhat irregular plateau over the experimental period with an average of about 30 ppm for males and about 45 ppm for females. There is no clear evidence of any progressive accumulation in fat during a 7-wk period. The fluctuations in the BHT concentrations in fat and liver (Fig. 2) during the feeding period show some similarities, but a further investigation would be necessary to establish this with certainty. At the termination of the feeding period the decrease in concentration in fat is more exponential, with a half-life period of 7–10 days. Throughout the whole period the apparent BHT content of normal rat fat and liver remained at a very low level. The extent to which metabolites of BHT would contribute to the concentrations reported in Table 1 and Figs. 1 and 2 is unknown, but it is certain that these figures will not include the conjugates described by Daere (1961). The figures obtained by colorimetric analysis show a higher concentration in fat than in liver, while the reverse is demonstrated by radioactivity; this is probably due to the presence of a greater proportion of conjugates in liver than in fat. Even in fat the colorimetric method gives values less than might be deduced from the evidence of the radioactive experiments; the daily dose of BHT received by the rats on the 0·5% diet approximates to 100 mg, and since Table 6 indicates that 0·054% of the dose should be present in 1 g fat 2 days later, it might be expected that the concentration in fat would exceed 100 ppm. The presence of BHT metabolites in fat is also suggested by the observation that radioactivity disappears from fat more rapidly than does the apparent BHT content obtained by chemical analysis.

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## Absorption et excrétion du butylate d'hydroxytoluène (BHT) chez le rat

**Résumé**—On a déterminé par une méthode colorimétrique la concentration du butylate d'hydroxytoluène (BHT; 3,5-di-*tert*-butyl-4-hydroxytoluène) dans les graisses et le foie de rats nourris avec des aliments contenant 0,5 et 1% de BHT pendant 50 jours. Dans l'un et l'autre de ces tissus, la concentration atteint rapidement un plateau fluctuant; dans le cas du régime à 0,5%, la concentration dans le tissu adipeux oscille autour de 30 ppm pour les mâles et de 45 ppm pour les femelles, tandis que dans le foie elle est beaucoup plus basse. Quand

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# Excretion of Butylated Hydroxytoluene (BHT) and Butylated Hydroxyanisole (BHA) by Man

J. W. DANIEL, J. C. GAGE, D. I. JONES and M. A. STEVENS

*Imperial Chemical Industries Limited, Industrial Hygiene Research Laboratories, Alderley Park, Cheshire, England*

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Abstract—After a single oral dose of [$^{14}$C]butylated hydroxytoluene (BHT) to man, about 75% of the administered radioactivity is excreted in the urine; with [$^{14}$C]butylated hydroxyanisole (BHA) the proportion is rather higher, in the region of 80–90%.

The kinetics of excretion is similar for both BHA and BHT. There is an early rapid excretion in which about 50% of the dose appears in the urine during the first 24 hr after dosing, followed by a slower phase which probably represents the release of the compounds or their metabolites stored in tissues.

The difference which has been observed between the relative rates of excretion of BHA and BHT by man and by the rat can probably be attributed to a difference between the metabolism of BHT in the two species.

## INTRODUCTION

The investigations of Golder, Ryan & Wright (1962) and of Ladomery, Ryan & Wright (1963) have indicated that the antioxidant butylated hydroxyanisole (2-*tert*-butyl-4-methoxyphenol; BHA) is rapidly excreted in urine by the rat, while butylated hydroxytoluene (2,6-di-*tert*-butyl-4-methylphenol; BHT) is excreted more slowly and incompletely by this route. This has led to the suspicion that BHT may be stored in the tissues and has caused it to be regarded with less favour for inclusion in food for human consumption. Daniel & Gage (1965) clearly demonstrated that there was no appreciable tissue retention of BHT in the rat, and that the incomplete recovery of the dose observed by the earlier workers was due to their failure to recognize the considerable faecal excretion, arising from the high proportion of BHT or its metabolites eliminated in the bile. Daniel & Gage (1965) also showed that the delay in the excretion of BHT by the rat was due to an enterohepatic circulation.

We have now studied and compared the excretion of radioactivity by man after administration of the two antioxidants, labelled with $^{14}$C.

## EXPERIMENTAL

*Materials.* BHT, randomly labelled with $^{14}$C in the methyl groups of the two *tert*-butyl groups, was obtained with a specific activity of 2·4 $\mu$C/mg from the New England Nuclear Corporation (Boston, Mass.). Thin-layer chromatography on silica-gel using petroleum ether (60–80°) as eluant indicated that it contained no radioactive impurities.

BHA, labelled with $^{14}$C in the methyl groups of the *tert*-butyl group, was prepared with the collaboration of Mr. G. H. Walker. It had a specific activity of 0·92 $\mu$C/mg and was shown radiochemically to be pure 2-*tert*-butyl-4-methoxyphenol; details of the synthesis and purification will be published elsewhere.

*Administration of dose.* Labelled BHT or BHA was weighed into a gelatin capsule and sufficient unlabelled material added to bring the total dose to 40 mg. This corresponds approximately to the figure of 0·5 mg/kg recommended by Joint FAO/WHO Expert Committee on Food Additives (1962) as the maximum acceptable daily intake for man. The amount of radioactivity administered to each of the four male subjects is shown in Table 1. 24-Hr samples of urine were collected from each subject into polythene bottles containing a little toluene, initially at daily intervals. Occasional samples of faeces were collected from subject A.

*Measurement of radioactivity.* Urine was examined by the method of Daniel & Gage (1965). Samples of faeces (approximately 0·1 g) were analysed by burning in a Hösli Pregl microanalytical combustion furnace, in a stream of oxygen. In order to minimize contamination due to absorption of $^{14}CO_2$ on copper oxide, the catalyst bed normally used for carbon and hydrogen determinations was modified by shortening the length of the copper oxide layer to 18 mm. The carbon dioxide produced was absorbed in 6 ml N-NaOH, and 2 ml of this solution was mixed with 17 ml of the scintillator solution for counting. Efficiency of counting was determined with a $Na_2{}^{14}CO_3$ internal standard.

## RESULTS

The radioactivity measurements on 24-hr urine samples, collected over an 11-day period from subjects A and B dosed with BHT and from subjects C and D dosed with BHA, are presented in Table 1 as percentages of the dose administered. Subsequent measurements were made on subject A who received the highest dose of radioactivity; on days 31, 56 and 79 after dosing the percentages found in a 24-hr sample were 0·2, 0·15 and 0·1 respectively. On day 100 the radioactivity of urine was not significantly above background.

Table 1. *Daily excretion of $^{14}C$ in urine of male subjects dosed with 40 mg of BHT or BHA, each labelled with $^{14}C$*

| Subject.... | A | B | C | D |
|---|---|---|---|---|
| Weight, kg.... | 88 | 100 | 73 | 80 |
| | BHT | | BHA | |
| Radioactivity administered, μC.... | 98 | 5 | 35 | 5·5 |
| Days after dosage | Percentage of dose excreted in urine | | | |
| 1 | 49·5 | 50·7 | 61·9 | 49·2 |
| 2 | 5·5 | 5·6 | 11·1 | 11·2 |
| 3 | 2·8 | 2·8 | 4·8 | 6·8 |
| 4 | 1·8 | 2·0 | 2·6 | 4·5 |
| 5 | 1·3 | 1·5 | 1·0 | 2·8 |
| 6 | 1·0 | 1·7 | 1·4 | 1·8 |
| 7 | 0·7 | 0·9 | 1·3 | 1·3 |
| 8 | | 0·5 | 0·8 | 0·9 |
| 9 | | 0·5 | 0·5 | 0·4 |
| 10 | | 0·7 | 0·7 | 0·6 |
| 11 | 0·6 | | 0·4 | |
| Total.... | 63·2 | 66·9 | 86·5 | 79·5 |

Measurements of radioactivity in faeces were made on subject A on days 10, 18 and 31 after dosing. The activity on these three days was found to correspond to 0·0015, 0·0007 and 0·0001% of the dose/g of faeces, or assuming the daily faecal output to be in the region of 200 g, to approximately 0·3, 0·15 and 0·02% of the dose per day.

## DISCUSSION

The results in Table 1 show that after a single dose of [$^{14}$C]BHT, at least two-thirds of the radioactivity is excreted in urine, with the major portion appearing within 24 hr of dosing. As occasional assays demonstrated a faecal excretion of about one-half of that in urine, it is probable that the remainder of the radioactivity was excreted by this route. As Daniel & Gage (1965) have shown that only about 25% of a dose of [$^{14}$C]BHT is excreted in the urine by male rats, spread over a period of 3–4 days, it is evident that BHT or its metabolites are eliminated more rapidly and to a greater extent in urine by man than by the rat. Daniel & Gage (1965) have also shown that the considerable faecal excretion of BHT in the rat, together with the delay in overall excretion, can be attributed to the presence of one or more metabolites in bile, leading to an enterohepatic circulation. An investigation into the metabolic products of BHT in the rat and in man is at present in progress in these laboratories.

The total urinary excretion of radioactivity of subjects C and D receiving an oral dose of BHA is greater than that of subjects A and B receiving BHT. The difference between the excretion of BHT and BHA is, however, less than would be expected from the comparison of these two substances by Golder *et al.* (1962) in the rat. As Astill, Mills, Fassett, Roudabush & Terhaar (1962) have demonstrated that the metabolism of BHA in man is similar to that in the rat, this is further evidence that the metabolic pathway of BHT is different in the two species. While the results in man suggest that there is some excretion of a BHT metabolite in bile, there is no evidence of an enterohepatic circulation.

With both BHT and BHA the bulk of the radioactivity appears on the first or second day after dosing, and thereafter a progressively diminishing slight excretion continues for a considerable period. Such a "tail" in the excretion may be attributable to a prolonged enterohepatic circulation, or to a slow release of the compounds or their metabolites from tissue storage. As the figures in Table 1 indicate that the kinetics of excretion is similar for both substances, and there is no suggestion that BHA can give rise to an enterohepatic circulation, tissue storage seems to be a more likely hypothesis. The release of material from tissues would probably follow first order kinetics, that is;

$$dU/dt = -dS/dt = KS \qquad (1)$$

Where U is the amount excreted in the urine, S is the amount in the body, and $S_o = U + S$. This equation may be transformed to present dU/dt as a function of t;

$$dU/dt = S_o k e^{-kt} \qquad (2)$$

If $u_t$ is the amount excreted in the time interval (t−1, t), then,

$$\log_e u_t = \log_e S_o(e^k - 1) - kt \qquad (3)$$

A plot of $\log_e u_t$ against t is shown in Fig. 1 with $u_t$ given the values of the daily urine excretion. It is clear from Fig. 1 that the excretion has an initial rapid phase followed by a slower phase. With subjects A, B and C the curves are very similar; for the second phase of excretion the value of k is about 0·12 and the value of $S_o$ can be calculated by equation (3) to be about 20% of the dose. That is, with these three subjects about one-fifth of the dose was retained in the tissues from which it was released with a half-life of about five days. The measured activity of the urine of subject A up to 79 days after dosing is in accord with a half-life of this magnitude. With subject D the value of k was about 0·4, with $S_o$ about 50% of the dose, that is, about one-half of the dose was retained in the tissues and released with a half-life of about two days. The cause of the greater tissue storage and more rapid release in this subject is not known; it may be due to a difference in the metabolic pathway.

According to Daniel & Gage (1965), the amount of radioactivity in rat tissues, excluding intestine, two and four days after an oral dose of [$^{14}$C]BHT, amounts to about 2·6 and

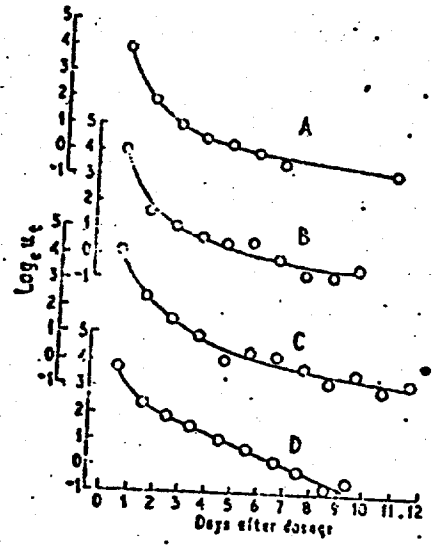


FIG. 1. Relationship between logarithm of daily excretion ($u_t$) and time. Subjects A and B received BHT; C and D, BHA.

1·7% of the dose respectively. Such figures are not available for BHA in the rat, but the available evidence suggests that they are not greater than this. The results in Table 1 suggest, therefore, that the tissue retention of both BHA and BHT in man is greater than in the rat.

From the above equations and the graph, the total urinary excretion by man may be estimated. With BHT a total of 75% of the dose may be expected with both subjects. With BHA, subject C would yield over 90% in urine, and subject D about 80%.

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**Excrétion d'hydroxytoluène butylé (BHT) et d'hydroxyanisol butylé (BHA) par l'homme**

*Résumé*—Après l'administration à l'homme d'une dose unique d'hydroxytoluène butylé (BHT) marqué au carbone-14, 75% environ de la radio-activité administrée sont excrétés dans l'urine; avec l'hydroxyanisol butylé (BHA) marqué au carbone-14, la proportion est plutôt plus élevée, voisine de 80-90%.

La cinétique de l'excrétion est similaire pour le BHA et le BHT. Au début il y a excrétion rapide, au cours de laquelle 50% environ de la dose apparait dans l'urine durant les 24 premières heures après l'administration, suivie d'une phase plus lente qui représente probablement la libération des produits ou de leurs métabolites emmagasinés dans les tissus.

La différence observée entre les vitesses relatives d'excrétion de BHA et de BHT par l'homme et le rat peut probablement être attribuée à une différence entre le métabolisme du BHT chez les deux espèces.

## Ausscheidung von butyliertem Hydroxytoluol (BHT) und butyliertem Hydroxyanisol (BHA) beim Menschen

Zusammenfassung—Nach einer einzelnen oralen, an Menschen verabreichten Dosis von [$^{14}$C]-butyliertem Hydroxytoluol (BHT) werden ungefähr 75% der aufgenommenen Radioaktivität im Urin ausgeschieden; bei [$^{14}$C]butyliertem Hydroxyanisol (BHA) liegt die Ausscheidung beträchtlich höher, im Bereich von 80–90%.

Die Kinetik der Ausscheidung ist bei BHA und BHT gleich. Es erfolgt eine frühe, rasche Ausscheidung, bei der etwa 50% der Dosis in den ersten 24 Stunden nach der Verabreichung im Urin erscheinen, woran sich eine langsamere Phase anschliesst, die wahrscheinlich die Freigabe der in den Geweben gespeicherten Verbindungen oder ihrer Stoffwechselprodukte darstellt.

Der beobachtete Unterschied zwischen den relativen Ausscheidungsgeschwindigkeiten von BHA und BHT beim Menschen und bei der Ratte kann wahrscheinlich einem Unterschied im BHT-Stoffwechsel bei den beiden Spezies zugeschrieben werden.

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# The Metabolism of 3,5-Di-*tert.*-butyl-4-hydroxytoluene in the Rat and in Man

By J. W. DANIEL, J. C. GAGE AND D. I. JONES
*Imperial Chemical Industries Ltd., Industrial Hygiene Research Laboratories, Alderley Park, Cheshire*

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1. The major metabolites of 3,5-di-*tert.*-butyl-4-hydroxytoluene (BHT) in the rat are 3,5-di-*tert.*-butyl-4-hydroxybenzoic acid (BHT-acid), both free (9% of the dose) and as a glucuronide (15%), and *S*-(3,5-di-*tert.*-butyl-4-hydroxybenzyl)-*N*-acetylcysteine. 2. The mercapturic acid does not appear to derive from the usually accepted enzymic mechanism, and may involve a non-enzymic reaction between BHT free radical and cysteine. 3. The ester glucuronide and mercapturic acid found in rat urine are also the major metabolites in rat bile and must be responsible for the enterohepatic circulation. 4. Free BHT-acid is the main component in rat faeces. 5. In man, BHT-acid, free and conjugated, is a minor component in urine, and the mercapturic acid is virtually absent. The bulk of the radioactivity is excreted as the ether-insoluble glucuronide of a metabolite in which the ring methyl group and one *tert.*-butyl methyl group are oxidized to carboxyl groups, and a methyl group on the other *tert.*-butyl group is also oxidized, probably to an aldehyde group. 6. These differences in metabolism by the rat and by man are sufficient to account for the difference in excretion by the two species.

The compound BHT* (butylated hydroxytoluene, I) has been the subject of extensive toxicological investigation on account of its use as an antioxidant in foods for human and animal consumption. During these studies, measurements of the excretion of radioactivity after the administration of [$^{14}$C]BHT to animals (Daniel & Gage, 1965) and to man (Daniel, Gage, Jones & Stevens, 1967) have revealed differences that may be due to a species variation in the metabolic fate of BHT.

Investigations into the metabolism of BHT after oral administration to the rabbit (Dacre, 1961; Ikagi & Aoki, 1962; Aoki, 1962) have shown the major metabolite in urine to be BHT-acid, formed by oxidation of the ring methyl group, and which is excreted both free and as a glucuronic acid conjugate. Another glucuronide has been detected but, on the basis of the infrared spectrum, has been identified as the conjugate of an alcohol produced either by oxidation of a *tert.*-butyl group or by oxidation of the ring methyl group. Using [$^{14}$C]-BHT, Daniel & Gage (1965) demonstrated that the delay in excretion that had been observed by earlier authors after the administration of an oral dose of BHT to rats was due to an enterohepatic circulation, and this was later confirmed by Ladomery, Ryan & Wright (1967*a*) after parenteral administration to rats. Ladomery, Ryan & Wright (1967*b*) claimed that the metabolites in rat bile after parenteral injection were somewhat similar to those in urine; they reported BHT-acid as an unspecified conjugate to be a major component, together with small amounts of the intermediate alcohol and aldehyde oxidation products, while another major metabolite remained unidentified. Daniel *et al.* (1967) produced evidence that the enterohepatic circulation observed in the rat did not occur in man, and they attributed this to a difference in metabolism by the two species. This has been the subject of the present investigation.

## MATERIALS AND METHODS

*BHT.* Unlabelled BHT (m.p. 70°) was obtained from Imperial Chemical Industries Ltd. (Heavy Organic Chemicals Division), Billingham, Co. Durham. It was stated to be not less than 99·5% pure. [$^{14}$C]BHT, randomly labelled in the methyl groups of both *tert.*-butyl groups, was obtained from the New England Nuclear Corp. (Boston, Mass., U.S.A.); the sample had a specific activity of 2·46$\mu$c/mg.; when chromatographed on thin-layer plates of silica gel with light petroleum (b.p. 60–80°) as eluent, only a single radioactive component was detected.

*3,5-Di-tert.-butyl-4-hydroxybenzyl alcohol* (*Ionox* 100).

* Abbreviations: BHT, 3,5-di-*tert.*-butyl-4-hydroxytoluene; BHT-acid, 3,5-di-*tert.*-butyl-4-hydroxybenzoic acid.

This was obtained from Shell Chemical Co. Ltd., Shell Centre, London.

*3,5-Di-tert.-butyl-4-hydroxybenzaldehyde.* This compound, m.p. 186–187°, was prepared by oxidation of BHT with bromine. The aldehyde was converted by a Cannizzaro reaction into very pale-yellow crystals of 3,5-di-*tert.*-butyl-4-hydroxybenzoic acid, m.p. 213° (Found: C, 72·3; H, 9·0. Calc. for $C_{15}H_{22}O_3$: C, 72·0; H, 8·8%).

*3,5-Di-tert.-butyl-4-hydroxylhippuric acid.* 3,5-Di-*tert.*-butyl-4-hydroxybenzoic acid was heated under reflux for 2hr. with glycine ethyl ester, dicyclohexylcarbodiimide and triethylamine in tetrahydrofuran (Sheehan & Hess, 1955). The solution was filtered and the filtrate evaporated to dryness, and the ethyl ester (m.p. 152°) was obtained by crystallization of the residue from benzene (Found: C, 68·1; H, 8·6; N, 4·6. Calc. for $C_{19}H_{29}NO_4$: C, 68·0; H, 8·7; N, 4·2%). The required acid (m.p. 189°) was obtained by hydrolysis of this ester with N-NaOH (Found: C, 65·5; H, 7·9; N, 4·5. Calc. for $C_{17}H_{25}NO_4$: C, 66·5; H, 8·1; N, 4·6%). When chromatographed on paper with the solvent systems C and F of Dacre (1961), $R_F$ values of 0·78 and 0·66 were obtained.

*S-(3,5-Di-tert.-butyl-4-hydroxybenzyl)-N-acetyl-L-cysteine.* To a solution of *N*-acetyl-L-cysteine (10m-moles) in methanol (50ml.) was added sodium methoxide (0·6g. of Na in 15ml. of methanol) followed by a solution of 3,5-di-*tert.*-butyl-4-hydroxybenzyl bromide (10m-moles) in ether (25ml.). The mixture was refluxed for 2hr. in an atmosphere of $N_2$, the solvent then removed under reduced pressure and the residue dissolved in water (25ml.). The aqueous solution was acidified with HCl, evaporated to dryness and the residue crystallized from ethyl acetate–light petroleum (b.p. 40–60°) to give white crystals, m.p. 135–137° (Found: C, 62·4; H, 7·6; N, 3·7; S, 8·1; $C_{20}H_{31}NO_4S$ requires C, 63·0; H, 7·9; N, 3·7; S, 8·4%).

*Collection of metabolites from the rat and from man.* Single oral doses (10–400mg./kg.) of [$^{14}$C]BHT were administered in olive-oil solution to groups of male Alderley Park strain specific-pathogen-free albino Wistar rats, weight 225–275g. Urine, faeces and bile were collected, as described by Daniel & Gage (1965). Samples of urine were collected from two human male subjects after a single oral dose (0·5mg./kg.) of [$^{14}$C]BHT administered in a gelatin capsule (Daniel *et al.* 1967). Details of the amount and radioactivity of the doses, and of the collection of the samples, are given in Table 2.

*Solvent fractionation of the metabolites.* The following procedure was used for the fractionation of urine and of bile, and for the further separation of metabolites liberated after hydrolysis of conjugates.

The sample, acidified to pH3, was continuously extracted with ether for 18hr. and, after separation of the ether extract, the aqueous layer containing the ether-insoluble metabolites was termed fraction F1. Acidic metabolites were separated by shaking the ether layer with aq. 5% (w/v) $Na_2CO_3$, and the ether layer containing neutral unconjugated metabolites, or neutral compounds formed by hydrolysis of ester sulphates during the ether extraction, was termed fraction F2. The $Na_2CO_3$ extract was acidified to pH6 and extracted with ether to give a fraction, F3, containing weakly acidic substances. The remaining aqueous layer was adjusted to pH3 and extracted with benzene, to give fraction F4 containing benzene-soluble acids, and then continuously with ether to give fraction F5 containing the remainder of the ether-soluble acids.

*Chromatography.* Fractions were chromatographed on thin-layer plates of silica gel G or alumina, and eluted with the solvent systems shown in Table 1. Radioactive areas were located by radioautography, with contact periods varying from 1 to 14 days. Non-radioactive reference compounds were detected by spraying with Gibbs reagent. For the purification of metabolites before identification, a solution was applied as a band to a thin-layer plate, and after development the radioactive area was located and removed, and the radioactive material was extracted from the support with methanol. The process was then repeated on another thin-layer plate with a different solvent system if necessary. The $R_F$ values and the colours with Gibbs reagent for the unlabelled reference samples are shown in Table 1.

Amino acids were separated by ascending chromatography on Whatman no. 1 paper, and located by spraying with ninhydrin, or by the azide–iodine reagent specific for sulphur-containing compounds (Chargaff, Levine & Green, 1948).

Acidic metabolites were separated on columns (2cm.× 15cm.) of DEAE-cellulose (Whatman DE-11 grade) and developed with methanolic solutions of acetic acid of increasing strength, or by gradient elution, with a mixing flask of 100ml. capacity.

*Measurement of radioactivity.* Liquid-scintillation counting of the biological samples and of their subsequent fractions was according to the methods described by Daniel & Gage (1965) and by Gage (1967).

**Table 1:** *Thin-layer chromatography of reference compounds*

Thin-layer chromatography was carried out on silica gel with the following solvent systems: *A*, butan-1-ol saturated with 2N-$NH_3$; *B*, butan-1-ol–ethanol–water (2:1:1, by vol.); *C*, butan-1-ol–acetic acid–water (12:3:5, by vol.); *D*, benzene–ethanol (9:1, v/v); *E*, chloroform; *F*, light petroleum (b.p. 60–80°).

| | Gibbs reagent colour | $R_F$ in solvent systems | | | | | |
|---|---|---|---|---|---|---|---|
| | | *A* | *B* | *C* | *D* | *E* | *F* |
| 3,5-Di-*tert.*-butyl-4-hydroxytoluene | Yellow | — | — | — | 0·77 | 0·92 | 0·43 |
| 3,5-Di-*tert.*-butyl-4-hydroxybenzyl alcohol | Magenta | — | — | — | 0·54 | 0·68 | — |
| 3,5-Di-*tert.*-butyl-4-hydroxybenzaldehyde | Magenta | — | — | — | 0·75 | 0·58 | — |
| 3,5-Di-*tert.*-butyl-4-hydroxybenzoic acid | Magenta | 0·5 | 0·82 | 0·90 | — | — | 0 |
| 3,5-Di-*tert.*-butyl-4-hydroxyhippuric acid | Magenta | 0·30 | 0·74 | 0·88 | — | — | — |
| *S*-(3,5-Di-*tert.*-butyl-4-hydroxybenzyl)-*N*-acetylcysteine | Yellow | 0·31 | 0·73 | 0·84 | — | — | — |

*Glucuronides.* Glucuronides were detected in samples and fractions by the naphtharesorcinol reaction. They were separated by precipitation with basic lead acetate, and the free acids were liberated by treating the precipitate with $H_2S$ (Kamil, Smith & Williams, 1952). The solution was filtered and concentrated under reduced pressure.

Glucuronides were hydrolysed either by heating under reflux with 2N-NaOH for 30min. or by incubation with 500 units of β-glucuronidase (Koch-Light Laboratories Ltd., Colnbrook, Bucks.)/ml. in 0·2M-acetate buffer, pH4·5, for 18–48hr. at 37°. The product after hydrolysis was subjected to the solvent fractionation process described above.

*Measurement of spectra.* Ultraviolet spectra were measured with a Unicam SP.800 recording spectrophotometer, and infrared spectra with a Perkin–Elmer 21 spectrometer with the sample either dispersed in liquid paraffin or in $CCl_4$ solution. Nuclear-magnetic-resonance spectra of metabolite solutions in $CDCl_3$ or perfluoroacetic anhydride were determined with a Varian HA-100 spectrometer. Mass spectra were obtained with an MS9 double-focussing spectrometer (Associated Electrical Industries Ltd., Manchester).

## RESULTS

The radioactivity in the various fractions of rat urine (four experiments) and bile and of human urine (two experiments), expressed as a percentage of the dose administered, is shown in Table 2.

### *Separation of metabolites from rat urine fractions*

*Fraction F1 (Expt. 1).* Concentrated hydrochloric acid (0·1vol.) was added to this fraction, which was then boiled under reflux in an atmosphere of nitrogen for 30min. Extraction with ether then removed 80% of the radioactivity, and the extract was shown by the fractionation procedure described in the Materials and Methods section to contain only neutral metabolites. Thin-layer chromatography (silica gel, solvent system *D*) revealed the presence of at least five substances.

*Fraction F2 (Expt. 1).* Thin-layer chromatography (silica gel, solvent system *D*) of the neutral metabolites in this fraction revealed five substances with $R_F$ values 0, 0·37, 0·41, 0·47 and 0·72.

*Fraction F3 (Expt. 2).* This fraction contained a single substance with $R_F$ 0·5 (silica gel, solvent system *A*). It was purified on silica-gel plates by using successively solvent systems *A* and *B*. The product (metabolite RU1) was crystallized from aqueous methanol (m.p. 203°).

*Fraction F4 (Expt. 1).* This fraction gave a strong reaction for amino acids after hydrolysis with 5N-hydrochloric acid, but only a weak reaction for glucuronides. Chromatography (silica gel, solvent system *C*) revealed one major metabolite ($R_F$ 0·87), which gave a yellow colour with Gibbs reagent, and two minor metabolites. The fraction was evaporated to dryness under reduced pressure and the residue dissolved in methanol and applied to a column of DEAE-cellulose. The column was eluted successively with methanol (20ml.), 0·1N-acetic acid in methanol (80ml.) and finally 0·3N-acetic acid in methanol. Fractions (10ml.) were collected at 30min. intervals; two fast-moving minor components containing 6 and 4% of the radioactivity were rejected and most of the radioactivity (87%) was collected in fractions 18–28. These fractions were combined and evaporated to dryness, and the residue was dissolved in ether and purified by thin-layer chromatography (silica gel, solvent systems *A*

**Table 2.** *Solvent fractionation of radioactivity in rat urine and bile, and human urine*

Radioactivity is presented as percentage of dose administered. Pooled bile from two rats was fractionated. See the text for meaning of fractions F1–F5.

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Dose (mg./rat or man) ... ... | 95·5 | 103·5 | 1·03 | 2·82 | 110 | 73 | 40 | 40 |
| Radioactivity (μc/rat or man) ... | 4·6 | 8·1 | 2·6 | 6·8 | 4·3 | 2·9 | 93 | 4·8 |
| No. in group ... ... ... ... | 9 | 4 | 5 | 5 | 1 | 1 | 1 | 1 |
| Duration of collection (hr.) ... ... | 72 | 96 | 96 | 72 | 48 | 48 | 24 | 10 |

| | Radioactivity (% of dose) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Rat urine | | | | Rat bile | | Human urine | |
| Expt. no. ... ... | 1 | 2 | 3 | 4 | | | 5 | 6 |
| Total radioactivity excreted | 57·5 | 58·5 | 10·1 | 24·4 | 25·7 | 30·0 | 49·5 | 46 |
| Radioactivity in fractions | | | | | | | | |
| F1 | 13·5 | 6·7 | 1·8 | 1·6 | 11·0 | | 40 | 33 |
| F2 | 0·9 | 1·7 | 1·0 | 1·0 | 0·7 | | 0·5 | 0 |
| F3 | 9·0 | 26·3 | 10·0 | 10·5 | 0·8 | | 1·0 | 1·0 |
| F4 | 13·0 | 10·8 | 2·4 | 5·8 | 2·9 | | 0·5 | 0·6 |
| F5 | 19·2 | 11·1 | 2·3 | 5·5 | 17·5 | | 7·0 | 10·6 |

and *B*). The solution obtained contained a single radioactive substance (metabolite RU2) with $R_F$ value 0·31 (silica gel, solvent system *A*).

*Fraction F5 (Expt. 1).* This fraction gave a strong positive reaction for glucuronides. Two radioactive metabolites were revealed by chromatography (silica gel, solvent system *C*) with $R_F$ values 0·67 and 0·73; these appear to be identical with the minor metabolites present in fraction F4. The fraction was evaporated to dryness and the residue dissolved in methanol and applied to a column of DEAE-cellulose. After elution with 60ml. of 0·1N-acetic acid in methanol, the column was subjected to gradient elution with 0·1N-acetic acid in methanol in the mixing vessel and 5N-acetic acid in methanol in the reservoir. Two metabolites were found in the 5ml. fractions; 13% of the radioactivity occurred in fractions 52–64, and 70% in fractions 72–96. The compounds in these two fractions were termed metabolite RU3 and metabolite RU4 respectively; both gave a positive reaction for glucuronides.

### *Separation of metabolites from human urine fractions*

*Fraction F1.* After hydrolysis of a portion of this fraction by boiling with 1vol. of 4N-sodium hydroxide for 30min., 85% of the radioactivity was obtained as ether-soluble acids. A similar conversion was obtained by hydrolysis with β-glucuronidase. Chromatography of the products (silica gel, solvent system *B*) showed two radioactive spots with $R_F$ values in the region of 0·5 and 0·65. Glucuronides were precipitated from the remainder of this fraction and hydrolysed with 2N-sodium hydroxide. The solution was adjusted to pH6 and shaken with ether; no radioactivity was extracted. The solution was then adjusted to pH3 and extracted again with ether for 18hr. The residue after evaporation of the ether was dissolved in methanol and fractionated on a column of DEAE-cellulose, with 0·1N-acetic acid in methanol as the eluent, 10ml. fractions being collected. A major component (compound RU1) containing 72% of the radioactivity added to the column was found in fractions 19–27, and a minor component in fractions 9–12 (18% of the radioactivity) and another in fractions 42–48 (8% of the radioactivity). The metabolite RU1 was purified by chromatography (silica gel, solvent system *B*).

*Fractions F2, F3 and F4.* These fractions from human urine contained only a very small amount of radioactivity and they were not further investigated. The radioactive components in fractions F3 and F4 appeared to be identical with the metabolites RU1 and RU2 in rat urine.

*Fraction F5.* This fraction gave a positive reaction for glucuronides. On hydrolysis with β-glucuronidase and subsequent solvent fractionation, 35% of the radioactivity remained with the acids extractable into ether at pH3, and 65% was in the neutral fraction. Chromatography (silica gel, solvent system *A*) showed that the acid fraction contained one component identical with metabolite RU1 by co-chromatography. The neutral fraction was separated into a number of components on silica gel (solvent system *D*).

### *Separation of metabolites from rat bile fractions*

*Fraction F3.* The small amount of radioactivity in this fraction was associated with one acid, which on co-chromatography (silica gel, solvent system *A*) appeared to be identical with metabolite RU1.

*Fraction F4.* Co-chromatography of this fraction indicated that the radioactive component present was identical with metabolite RU2.

*Fraction F5.* This fraction gave a positive reaction for glucuronic acid. It was purified by chromatography on silica gel (solvent system *A*). A portion of the purified metabolite was hydrolysed with sodium hydroxide and then solvent fractionated; 77% of the radioactivity was associated with an acid that on co-chromatography appeared to be identical with metabolite RU1, 4% appeared in the neutral fraction and 19% appeared to be unhydrolysed. After enzyme hydrolysis the amounts of metabolite RU1, neutral material and unhydrolysed material were 53, 8 and 39% respectively.

### *Identification of metabolites*

*Metabolite RU1.* The ultraviolet spectrum of this metabolite had an absorption maximum at 257mμ and a minimum at 238mμ, and was identical with that of synthesized 3,5-di-*tert.*-butyl-4-hydroxybenzoic acid (III), and the $\epsilon_{max.}$ 11000 was very close to the value 10000 for the authentic material. Co-chromatography (silica gel, solvent system *A*) confirmed the identity and the m.p. of the authentic material was not depressed by the addition of metabolite RU1. The measured specific radioactivity of metabolite RU1 was 0·011μc/mg., compared with the theoretical value of 0·043μc/mg. (Found: C, 72·0; H, 8·9. Calc. for $C_{15}H_{22}O_3$: C, 72·0; H, 8·8%).

*Metabolite RU2.* Although this compound gave a reaction for amino acids on hydrolysis, its $R_F$ value 0·3 (silica gel, solvent system *A*) and the yellow colour produced on the chromatogram with Gibbs reagent clearly indicated that it was not 3,5-di-*tert.*-butyl-4-hydroxyhippuric acid. Boiling with 1vol. of conc. hydrochloric acid for 4hr. in an atmosphere of nitrogen yielded a yellow neutral compound with $R_F$ 0·79 (silica gel, solvent system *A*). Ionox 100 by the same acid treatment also yields a yellow neutral compound with the same $R_F$ value. The acid hydrolysate also contains two sulphur-containing amino acids, detected on paper by

Table 3. *Comparison of nuclear-magnetic-resonance spectrum of metabolite RU 2 with that of S-(3,5-di-tert.-butyl-4-hydroxybenzyl)-N-acetylcysteine*

| Metabolite RU2 | | | Authentic sample | | | |
|---|---|---|---|---|---|---|
| τ | Pattern | Relative intensity | τ | Pattern | Relative intensity | Proton assignment |
| 2·96 | Singlet | 2 | 0·14 | Broad singlet | 1 | Carboxyl |
| 3·68 | Doublet | 1 | 2·98 | Singlet | 2 | Aromatic |
| 4·4 | Broad singlet | 1 (+1) | 3·60 | Doublet | 1 | Amide |
| | | | 4·90 | Broad singlet | 1 | Carboxyl (+ hydroxyl) |
| 5·35 | Multiplet | 1 | 5·40 | Multiplet | 1 | Hydroxyl |
| 6·36 | Singlet | 2 | 6·40 | Singlet | 2 | Cysteine methine |
| 7·10 | Doublet | 2 | 7·12 | Doublet | 2 | *S*-Benzyl methylene |
| 8·04 | Singlet | 3 | 8·05 | Singlet | 3 | Cysteine methylene |
| 8·60 | Singlet | 18 | 8·62 | Singlet | 18 | *N*-Acetyl methyl |
| | | | | | | *tert.*-Butyl methyl |

spraying with ninhydrin and iodine–azide reagent; these were identified as cystine and cysteine by co-chromatography. These observations suggested a mercapturic acid derived from BHT. The nuclear-magnetic-resonance measurements are shown in Table 3; the presence of the two-proton aromatic singlet and a singlet of τ 6·38 (methylene adjacent to an aromatic ring and a sulphur atom) suggested that metabolite RU 2 was *S*-(3,5-di-*tert.*-butyl-4-hydroxybenzyl)-*N*-acetylcysteine (V). The mass spectrum of metabolite RU2 exhibited a molecular ion at *m/e* 381, which by accurate mass measurement was found to correspond to the elemental composition $C_{20}H_{31}NO_4S$ required for the proposed mercapturic acid conjugate. Confirmation of this structure was obtained by co-chromatography of the metabolite with an authentic sample, and also by comparison of the nuclear-magnetic-resonance and mass spectra (Table 3). The infrared spectra of the metabolite and of the synthesized material both showed absorption maxima at 3600, 3300, 2500, 1730, 1650, 1530 and 1310 cm.$^{-1}$, and their ultraviolet spectra both showed absorption maxima at 233 and 276 mμ, with a shoulder at 250–283 mμ.

*Metabolite RU 3.* The solution of this metabolite was evaporated to dryness under reduced pressure, and the residue dissolved in acetate buffer and subjected to hydrolysis with β-glucuronidase. Subsequent solvent fractionation revealed 95% neutral compounds. Chromatography (alumina, solvent system *E*) showed several radioactive compounds, a minor one of which was probably Ionox 100 (II).

*Metabolite RU 4.* After hydrolysis with β-glucuronidase and solvent fractionation, a single weakly acidic substance was recovered. This gave an ultraviolet spectrum identical with that of metabolite RU1, and on chromatography (silica gel, solvent system *A*) the two substances migrated together. Metabolite RU4 was therefore identified as the ester glucuronide of 3,5-di-*tert.*-butyl-4-hydroxybenzoic acid (III).

*Metabolite RU 1.* The evidence obtained during the separation of this metabolite indicated that it was excreted in urine as an ester glucuronide. On hydrolysis of this glucuronide with 2N-sodium hydroxide an acid was obtained that was extractable into ether at pH 3 but not at pH 6, and was therefore stronger than the acid produced by oxidation of the ring methyl group of BHT. Nevertheless, its ultraviolet spectrum, with a maximum at 254 mμ and a minimum at 231 mμ, was very close to that of BHT-acid, suggesting that metabolite RU 1 was a benzoic acid derivative with a second carboxyl group responsible for the higher acidity. Evidence of two carboxylic groups was also obtained from the infrared spectrum. The product was methylated with diazomethane and further purified on silica gel. The mass spectrum demonstrated a molecular ion at *m/e* 322, and accurate measurement gave the formula $C_{17}H_{22}O_6$. The nuclear-magnetic-resonance spectrum showed two peaks in the aromatic region at τ 2·4 and 2·5, two peaks assigned to methoxyl resonances at τ 6·10 and 6·36, and two peaks (aliphatic methyl) at τ 8·40 and 8·71; the relative intensities of these six peaks were approximately 1:1:3:3:6:6. The absence of a signal of relative intensity 3 at approximately τ 8·0 indicated the absence of a ring methyl group, and the presence of two distinct aromatic signals and two peaks of relative intensity 6 indicated that both *tert.*-butyl groups had been attacked but not in the same manner. The two methoxyl groups could be attributed to methylation of carboxyl groups, one deriving from oxidation of a ring methyl group and the other from oxidation of a *tert.*-butyl methyl group. There was no direct evidence for the nature of the change in the second *tert.*-butyl group, but, on the basis of the formula given above, the only

possibility is the oxidation of a methyl group to an aldehyde group. No signal for a formyl proton was observed between $\tau$ 0·0 and 1·0, but it is possible that a signal at $\tau$ 4·6 is derived from a hydrated aldehyde group. This is not in conflict with an *m/e* value of 322, for a water molecule would be lost under the conditions of measurement. This evidence indicates that metabolite HU1 is 4-carboxy-2-(1-carboxy-1-methylethyl)-6-(1-formyl-1-methylethyl)phenol, possibly as a hydrate.

*Neutral material.* Thin-layer chromatography (silica gel, solvent system *D*) of neutral fractions, both free and after hydrolysis, revealed the presence of up to five compounds, none of which corresponded to BHT, Ionox 100 or 3,5-di-*tert.*-butyl-4-hydroxybenzaldehyde. An acetylated derivative of an ether glucuronide extracted from rabbit urine was supplied to us by Dr J. C. Dacre, which he claimed (Dacre, 1961) to be methyl [$\beta'$-(3-*tert.*-butyl-2-hydroxy-5-methylphenyl)-$\beta'\beta'$-dimethyl 2,3,4-tri-*O*-acetyl-$\beta$-D-glucopyranosid]uronate; this was subjected to acid hydrolysis, but the product did not appear to be identical with any component of the neutral fraction. These neutral products are unstable during isolation; BHT is known to give a variety of compounds on mild oxidation, and this investigation has shown that Ionox 100 is readily degraded when heated in acid in the absence of oxygen. As some of the neutral metabolites detected may therefore be artifacts, and each constitutes only a small proportion of the dose, they were not further investigated.

### *Rat faeces acetone extract*

From chromatographic evidence the major metabolite in this fraction was metabolite RU1, and there was also a small amount of radioactive material with $R_F$ about 0·43 (silica gel, solvent system *F*) that on co-chromatography appeared to be BHT.

## DISCUSSION

The qualitative information on the metabolites in rat and human urine, and in rat bile, is collected in Table 4, together with an estimate of the amounts present. The major metabolite in rat urine is the acid produced by oxidation of the ring methyl group (BHT-acid, III), which is excreted both free and conjugated with glucuronic acid. This is in accord with Dacre's (1961) observations in the rabbit, although the glycine conjugate, which he claimed to be excreted by that species, does not appear in rat urine. Dacre's (1961) own evidence for this metabolite is not entirely satisfactory, as a sample of the authentic glycine conjugate, when subjected to chromatography under the conditions that he described, gives $R_F$ values different from those that he claimed. Doubt about Dacre's (1961) evidence has also been expressed by Wright, Akintonwa, Crowne & Hathway (1965), who failed to find this conjugate in their study of the metabolism of Ionox 100 by the rat and the dog. BHT-acid can be extracted by ether at pH 6; this suggests that it is a very weak acid, and possibly too weak to form the acyl-CoA derivative, which is stated by Schachter & Taggart (1954) to mediate the glycine conjugation of benzoic acid derivatives.

The second major metabolite of BHT in the rat is a mercapturic acid (V), which appears to be formed by a mechanism not previously described. Although aromatic hydrocarbons are known to yield mercapturic acids through some intermediate oxidation product (Boyland & Sims, 1958), this has not been reported for aliphatic hydrocarbons or for alkyl substituents on aromatic rings. Ionox 100 (Wright *et al.* 1965) does not form a mercapturic acid, and though a benzyl mercapturic acid is known to be

Table 4. *Metabolites in rat urine and bile, and in human urine*

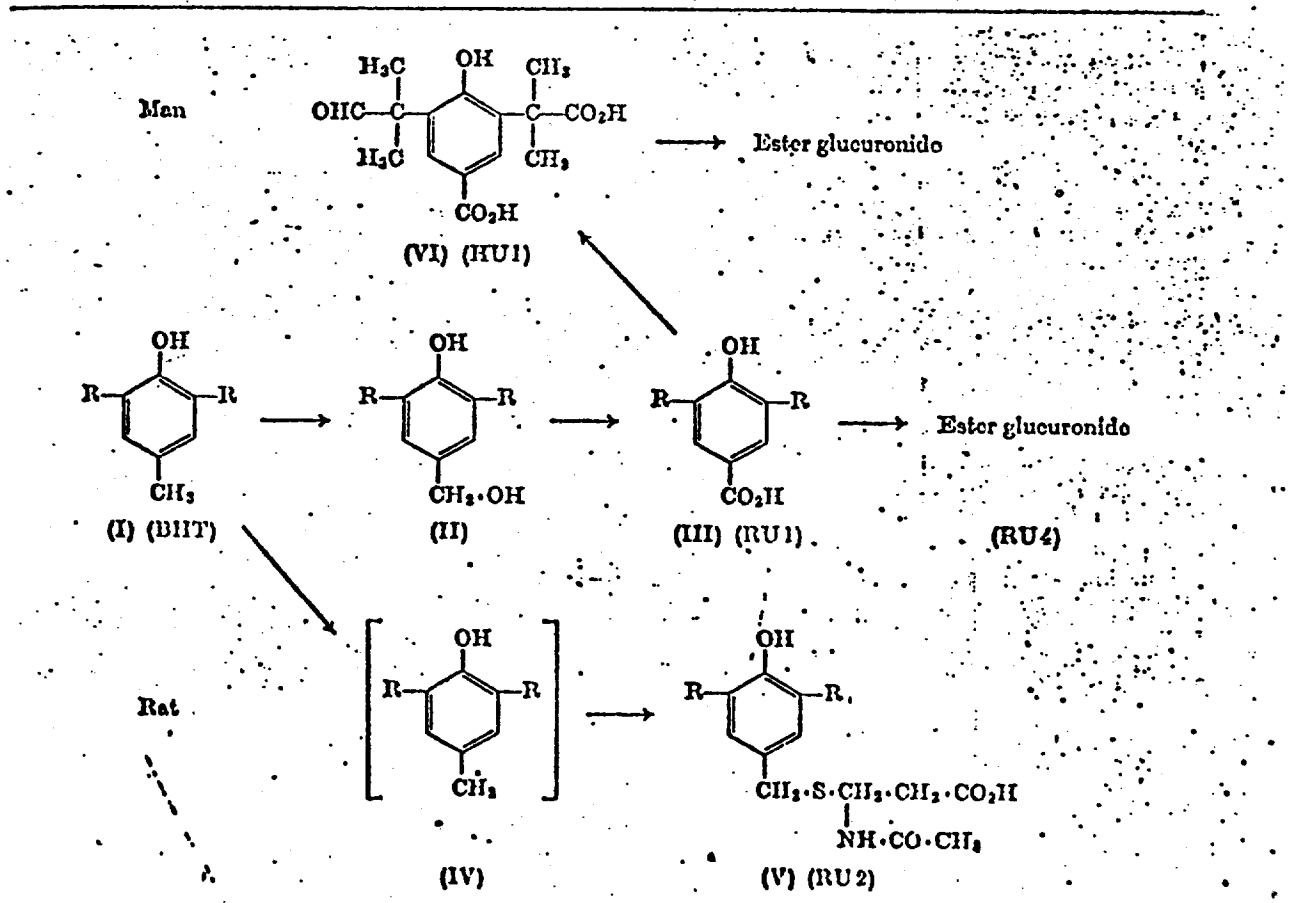
Rat urine results are based on Expt. 1, human urine on Expt. 5 (Table 2). Figures represent percentage conversion of BHT administered.

| Metabolite | Conversion of BHT (%) | | |
|---|---|---|---|
| | Rat urine | Rat bile | Human urine |
| 3,5-Di-*tert.*-butyl-4-hydroxybenzoic acid (III) | 9 | 0·8 | 1 |
| (3,5-Di-*tert.*-butyl-4-hydroxybenzoyl $\beta$-D-glucopyranosid)uronic acid | 15 | 14 | 2 |
| *S*-(3,5-Di-*tert.*-butyl-4-hydroxybenzyl)-*N*-acetylcysteine (V) | 11 | 3 | — |
| 4-Carboxy-2-(1-carboxy-1-methylethyl)-6-(1-formyl-1-methylethyl)phenol (? hydrate) (VI) as ester glucuronide | — | — | 35 |
| Neutral compounds | | | |
| Free | 1 | 0·7 | 0·5 |
| Glucuronides | 4 | 2 | 5 |
| Ester sulphates (?) | 13·5 | — | — |

formed from benzyl chloride (Stekol, 1938), there is no precedent for an intermediate product of this nature in the metabolism of substituted phenols. Mercapturic acid formation by glutathione *S*-alkyltransferase (Johnson, 1966) is limited to alkyl halides. The mechanism involving side-chain epoxidation, proposed by James & Jeffery (1961) and James & White (1967), appears to be ruled out on structural grounds. Moreover, the formation of the mercapturic acid is not attended by hydroxylation elsewhere in the molecule. A more likely mechanism involves the free radical (IV), which appears to be formed when BHT accepts a single electron, and which is considered to be involved in the antioxidant action of this compound (Cosgrove & Waters, 1951); this free radical may then react with cysteine by a non-enzymic mechanism and the product acetylated to yield a mercapturic acid.

The amount of neutral metabolites excreted by the rat is small, the total excretion as free and conjugated derivatives being less than 10%. In this context, compounds containing the BHT phenolic group are classed as neutral, as the steric effect of the adjacent *tert.*-butyl groups inhibits phenolic properties. This low excretion is contrary to observations in the rabbit, and there is no evidence in the rat of the excretion of a BHT metabolite hydroxylated at a *tert.*-butyl group (Dacre, 1961), though there is some evidence of hydroxylation at the ring methyl group (Ionox 100, II), presumably an intermediate stage in the formation of BHT-acid. There is little point in undertaking a further investigation of these neutral compounds as they readily undergo change when processed. When Ionox 100 is boiled with dilute acid it is rapidly degraded to a variety of products, and it is possible that this compound may be the major neutral metabolite excreted in urine.

The metabolites found in rat bile consist almost entirely of the conjugates occurring in urine, the bulk of the radioactivity being due to the glucuronide of BHT-acid, but significant amounts of the mercapturic acid and of glucuronides of neutral metabolites are also present. Unconjugated metabolites are almost entirely absent, confirming the view that only water-soluble conjugates are excreted in bile. All of the derivatives found in bile are also lipid-soluble and only weakly acidic, and




Scheme 1. Metabolism of BHT in the rat and in man (R represents *tert.*-butyl).

are therefore capable of being reabsorbed by the gut to give an enterohepatic circulation. The occurrence of unconjugated BHT-acid in faeces does not necessarily indicate that the glucuronide of this metabolite must be broken down in the gut before it can be reabsorbed.

The results in Table 2 suggest that at the lower dose of BHT there is a lower content of metabolites in the urine associated with a lower excretion of conjugates; in these experiments the partition of conjugates between bile and urine appears to have been in favour of the bile. However, an earlier investigation (Daniel & Gage, 1965) has indicated that high urinary excretion in the rat may occur with low BHT dosage.

The metabolism of BHT in man is very different from that which occurs in the rat. The amount of BHT-acid and its glucuronide in urine is very small, and only a trace of the mercapturic acid derivative is present. In place of these ether-soluble derivatives, the major metabolite is an ether-insoluble glucuronide of a BHT oxidation product, in which all three alkyl substituents have been attacked (VI). Although this structure has not been confirmed by independent synthesis, it is very probable that oxidation of the ring methyl group and a *tert.*-butyl group has produced a dicarboxylic acid, and the second *tert.*-butyl group has been oxidized to an aldehyde group, possibly masked by hydration.

On the basis of these observations, the reactions shown in Scheme 1 may be proposed for the metabolism of BHT in the rat and in man. It seems very probable that the enterohepatic circulation in the rat, with attended delayed urinary excretion, derives from the biliary excretion of BHT-acid glucuronide, and possibly also of the BHT mercapturic acid. The rapidity of the first phase of urinary excretion in man, in which about one-half the dose appears within 24hr. (Daniel *et al.* 1967), indicates that there is no considerable enterohepatic circulation in man, and, if the glucuronide of compound (VI) is responsible for the faecal excretion in man, it cannot be readily reabsorbed from the gut. A little BHT-acid glucuronide is found in human urine and it is possible that an enterohepatic circulation occurs to a minor extent. It is conceivable that the second phase of slow urinary excretion in man, attributed by Daniel *et al.* (1967) to a slow release from the tissues, may be due to such a circulation; if so, absorption from the gut and excretion through the liver must be very efficient to lead to the observed half-life in the body.

The results obtained in this investigation are sufficient to account for the observed differences in the kinetics of BHT excretion in the rat and in man, though they give no indication whether the pharmacological effects of BHT in the two species are likely to be different.

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# THE EFFECT OF THE ANTIOXIDANT BUTYLATED HYDROXY TOLUENE ON SERUM LIPID AND GLYCOPROTEIN LEVELS IN THE RAT

A. J. DAY, A. R. JOHNSON, M. W. O'HALLORAN[1] AND C. J. SCHWARTZ[2]

(From the Commonwealth Antioxidant Research Project and the Department of Human Physiology and Pharmacology, University of Adelaide).

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## SUMMARY.

Observations were made of the effects of variations in the dietary content of lard and butylated hydroxy toluene (BHT) on total serum cholesterol, phospholipid, total esterified fatty acid, mucoprotein and hexosamine levels of male rats in a paired feeding experiment, and of female rats in a chronic study.

A concentration of 0·001 p.c. BHT (dry weight) caused no changes in any of the serum constituents studied.

0·1 p.c. BHT, with 10 p.c. lard supplement to the diet, increased the serum cholesterol levels of female rats in the chronic study, but no other significant changes were produced.

0·5 p.c. BHT produced increases in the serum cholesterol levels of male rats within five weeks, and serum cholesterol, phospholipid and mucoprotein levels in female rats after eight months.

A 10 p.c. and 20 p.c. lard supplement to the basic diet produced significant reductions in the serum cholesterol, phospholipid and hexosamine levels of *ad lib.* fed male rats within five weeks.

Restriction of food intake during the first two weeks, on a basic diet unsupplemented with lard, produced significant reductions in serum cholesterol and phospholipid levels in male rats after five weeks.

The serum levels of total esterified fatty acids were not affected by any of the treatments applied.

A possible relationship between the metabolism of relatively large doses of BHT, the fatty acids and the transport of cholesterol and phospholipid is suggested.

## INTRODUCTION.

Butylated hydroxy toluene (2:6-di-*t*-butyl-p-cresol, referred to as BHT) was introduced as an antioxidant to delay the onset of rancidity in foodstuffs for human consumption (Dugan *et al.*, 1954). Although this antioxidant has been

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[1] Present address: Nicholas Institute of Medical and Veterinary Research, Burnham [illegible], Victoria.

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In use for some time, very little information is available concerning its metabolism *in vivo*. It has been shown that a proportion of an ingested dose of BHT is excreted in the urine as glucuronides and ethereal sulphates (Deere, 1957; Deichmann *et al.*, 1955). Johnson *et al.* (1955a) have shown that in the rat a small percentage of ingested BHT is probably deposited in the perirenal fat increasing its chemical stability, and reducing its iodine value.

The present paper describes the effect of BHT on serum lipid and glycoprotein levels, and suggests that relatively large doses of BHT interfere with the metabolism of those fatty acids involved in the transport of both total serum cholesterol and phospholipid.

## Materials and Methods.

*Paired feeding experiment.*

Forty-five pairs of male weanling litter mates, of the Norway Hooded strain of rat, were selected from litters of our stock colony, on a basis of the closest possible approximation of their weights.

The 45 pairs were randomly subdivided into five groups, avoiding the use of two litter pairs from the same original litter within the same group. The five groups represented five replicates within the experimental design. These replicates were treated as a unit, i.e. they were sacrificed and serum samples taken and analysed under the same conditions, to ensure valid comparisons.

The animals were housed singly, one of the sibs of each pair being pair-fed against its litter mate. The nine litter pairs within one group were randomly allocated to the nine pairs of diets within one replicate (Table 1).

TABLE 1.

*Lard and BHT content of diets.*

| Percentage lard supplement to stock diet (by weight) | Percentage BHT | | Percentage BHT | | Percentage BHT | |
|---|---|---|---|---|---|---|
| | *Ad lib.* feeding | Pair-fed | *Ad lib.* feeding | Pair-fed | *Ad lib.* feeding | Pair-fed |
| 0 | 0 | 0·001 | 0·1 | 0 | 0·5 | 0 |
| 10 | 0[a] | 0·001 | 0·1[a] | 0 | 0·5[a] | 0 |
| 20 | 0[a] | 0·001 | 0·1[a] | 0 | 0·5[b] | 0 |

(a) and (b) Diets also used in chronic study.

A further nine weanling male litter pairs, of the Albino Wistar strain of rat, all of approximately the same weight, were randomly allocated to the nine groups within the experiment. The results from these 18 animals did not differ significantly from those of the Norway Hooded strain and the results of both strains of rat were pooled for statistical analyses.

The conventional methods of the paired feeding technique were employed, the animals within the first cage of each diet pair (Table 1) being fed *ad lib.* and their food consumption measured to 0·1 gm. On the succeeding day its litter mate was fed the same amount of the appropriate diet.

The stock diet and the method of preparation of the experimental diets containing 10 and 20 p.c. lard supplement have been described previously (Johnson *et al.*, 1953a). The BHT[3] was added to the diet unsupplemented with lard by dry homogenisation in a Waring blender in the appropriate proportions of BHT, full cream powdered milk, sodium chloride, and calcium carbonate. The blended material was then mixed with the remainder of the dry materials of the diet, and treated in a similar fashion to the lard-containing diets.

The concentrations of BHT in the lard were so chosen that direct comparisons could be made between the effect of varying amounts of BHT, at different levels of lard supplement to the stock diet, and conversely the effect of a varying lard content of the diet at the same level of BHT.

Five weeks after they were placed on their respective diets, approximately 5 ml. of blood were taken by intracardiac puncture under ether anaesthesia, from the first 18 animals representing nine litter pairs. The animals were then sacrificed by further ether anaesthesia. The clotted blood was centrifuged at 3,000 r.p.m. for 10 minutes. The sera were analysed in duplicate for total cholesterol (Zlatkis *et al.*, 1953), phospholipid (Brown, 1954), and total esterified fatty acids (Stern and Shapiro, 1953). In these determinations 3:1 ethanol ether extracts of the sera were used.

Mucoprotein was determined by the method described by Winzler *et al.* (1948), and hexosamine by the method of West and Clarke (1953). Both methods have been modified as previously described by Schwartz and Gilmore (1956).

Subsequently the other groups of 18 animals were sacrificed at approximately three-day intervals. Thus the time that the animals were on a given diet ranged from five to eight weeks. No differences were found between the sera constituents in the first and last groups.

*Chronic study.*

A second experiment to evaluate the effects of continuous ingestion of BHT in female rats over an eight-month period, was designed as follows. One batch of three weanling female litter mates, of the Norway Hooded strain of rat, were randomly distributed between the 10 p.c. lard supplemented diets containing 0, 0·1 and 0·5 p.c. BHT (see a, Table 1), and a second between the 20 p.c. lard supplemented diets containing the same amount of BHT (see b, Table 1). This procedure was repeated eight times, so that on each diet there were eight animals caged in two groups of four. In all experiments to be described the various litters were treated as a unit to ensure valid comparisons.

The diets were supplied *ad lib.* during the whole course of the experiment. After five months of treatment the animals were mated with similarly fed litter mates, as part of a reproduction study (Johnson and Williams, [illegible]). At least six weeks after weaning of the young, the female rats were sacrificed and the sera examined as outlined above.

## RESULTS.

*Paired feeding experiment—Control animals.* On each lard level there were three groups of control animals: those being fed *ad lib.*, those being fed the same amount of food as their litter mates whose diet contained 0·1 p.c. BHT, and similarly those pair-fed control animals whose diet contained 0·5 p.c. BHT. Thus, the only differences between the groups of control animals on the same lard supplement were in the amounts of food consumed, and any other effects arising from the pair-fed food supply. The differences were determined by the effect of BHT on the food consumption of their litter mates. The pair-fed controls consumed virtually the same amounts of food as did those animals whose diet contained BHT.

[3] Tenox-BHT, [illegible]

During the first week, the introduction of 0·1 p.c. and 0·5 p.c. BHT to the diet significantly reduced ($p < 0{\cdot}001$) the food consumption irrespective of the level of lard supplementation. By the fifth week there were no significant differences in the food consumption even when the diet contained 0·5 p.c. BHT (Fig. 1), the rats presumably having become "adapted" to the hot and peppery nature of the BHT-containing diets.

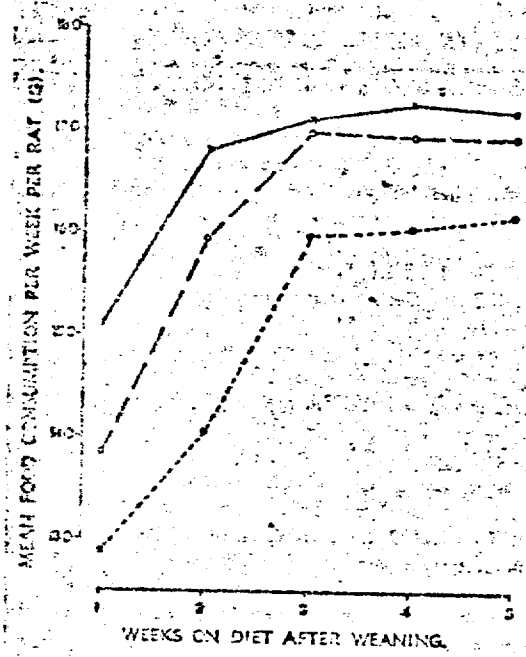


Fig. 1. *The effect of dietary BHT upon the food consumption of male weanling rats.*

×———× 0·0 p.c. BHT.
○- - - -○ 0·1 p.c. BHT.
•. . . .• 0·5 p.c. BHT.

Note: Standard error of a mean (S.E.M.) for week 1 = 2·1 on 20 degrees of freedom (d.f.). S.E.M. for week 5 = 4·3 on 20 d.f.

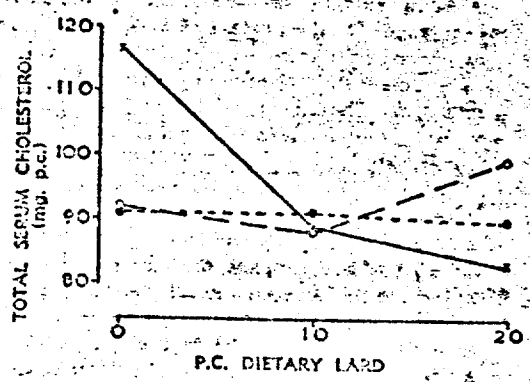


Fig. 2. *The effect of lard and pair-feeding against animals whose diet contained BHT, on the total serum cholesterol levels of male rats.*

×———× *Ad lib.* feeding.
•- - - -• Pair-fed against animals whose diet contained 0·1 p.c. BHT.
○. . . .○ Pair-fed against animals whose diet contained 0·5 p.c. BHT.

S.E.M. = 5·1 on 20 d.f.

The serum mucoprotein and fatty acid levels of the control animals were constant, irrespective of the variation in diet and food intake. However, the total serum cholesterol, phospholipid and hexosamine levels differed significantly between control rats.

*Total serum cholesterol* (Fig. 2 and Table 2). The supplementation of the stock diet by both 10 p.c. and 20 p.c. lard caused similar reductions in the serum cholesterol levels of *ad lib.* fed male rats after five weeks. No such reductions in the serum cholesterol levels of control rats were observed when the food intake was restricted during the first one or two weeks of the experiment [illegible]

TABLE 2.

*The effect of BHT, lard and pair-feeding on total serum cholesterol levels in the male rat.*

| | | | Percentage dietary BHT | | |
|---|---|---|---|---|---|
| | | | ·001 | ·1 | ·5 |
| Percentage dietary lard | 0 | Treated | 102·7[a] | 109·5 | 112·5 |
| | | Control | 116·5 | 92·0 | 91·2 |
| | | Difference | − 13·8 | 17·5 | 21·3 |
| | 10 | Treated | 97·7 | 94·0 | 126·1 |
| | | Control | 89·0 | 88·3 | 90·8 |
| | | Difference | 8·7 | 5·7 | 35·3 |
| | 20 | Treated | 80·7 | 95·0 | 142·4 |
| | | Control | 83·4 | 100·2 | 90·2 |
| | | Difference | − 2·7 | − 5·2 | 52·2 |

(a) Mean total serum cholesterol (mg. p.c.) of six animals.

Note: Standard error of mean (S.E.M.) of differences is 10·1 on 20 degrees of freedom (d.f.).

sequent on the paired feeding of the control animals against animals whose diet contained 0·1 p.c. and 0·5 p.c. BHT.

The restriction of the food intake itself had an effect on the serum cholesterol levels of the control animals, and this effect was dependent upon the extent of dietary lard supplementation. Thus a reduction in the level of serum cholesterol was observed when the food was restricted and no lard present in the diet. Under similar conditions of food restriction no alterations were noted when the diet contained 10 p.c. lard, and a raised serum cholesterol was observed with a 20 p.c. lard supplement.

TABLE 3.

*The Effect of BHT, lard and pair-feeding on serum phospholipid levels in the male rat.*

| | | | Percentage dietary BHT | | |
|---|---|---|---|---|---|
| | | | ·001 | ·1 | ·5 |
| Percentage dietary lard | 0 | Treated | 131·2[a] | 126·8 | 130·2 |
| | | Control | 141·0 | 117·8 | 122·0 |
| | | Difference | − 9·8 | 19·0 | 17·2 |
| | 10 | Treated | 124·2 | 115·5 | 157·7 |
| | | Control | 116·5 | 119·5 | 120·3 |
| | | Difference | 7·7 | − 4·0 | 37·3 |
| | 20 | Treated | 108·0 | 128·0 | 173·3 |
| | | Control | 109·0 | 131·0 | 123·3 |
| | | Difference | − 1·0 | − 3·0 | 50·0 |

(a) Mean serum phospholipid (mg. p.c.) of six animals.

Note S.E.M. of differences is 12·2 on 20 d.f.

*Serum phospholipid* (Table 3). The changes of the serum phospholipid of the control rats, upon supplementation of the diet with lard or restriction of the food intake, closely paralleled those of the serum cholesterol.

*Serum hexosamine* (Table 4). Supplementation of the stock diet by 10 p.c. and 20 p.c. lard produced similar decreases in the serum hexosamine of the control animals.

TABLE 4.

*Effect of lard feeding on the serum hexosamine content in male rats.*

| | Percentage lard supplement to stock diet | | |
|---|---|---|---|
| | 0 | 10 | 20 |
| Hexosamine mg./100ml. | 129[a] | 121($p<0{\cdot}01$[b]) | 122($p<0{\cdot}01$) |

(a) Figures quoted represent means of 18 animals.

(b) p is probability that treatments differ significantly from control.

Note: S.E.L.L. = 2·2 on 20 d.f.

*The effect of BHT on serum constituents.*

*Paired feeding experiment.* In order to eliminate the effects of the significant differences which occurred between groups of control animals, an analysis of variance was made on the difference between each litter pair. BHT at a dietary level of 0·5 p.c. increased the serum cholesterol levels of male rats within five weeks, even when any effects of BHT on the food consumption were eliminated by the use of the paired feeding technique. The increases in serum cholesterol levels were dependent upon the degree of lard supplementation (Table 2 and Fig. 3), an increase in the lard content of the diet enhancing the elevation

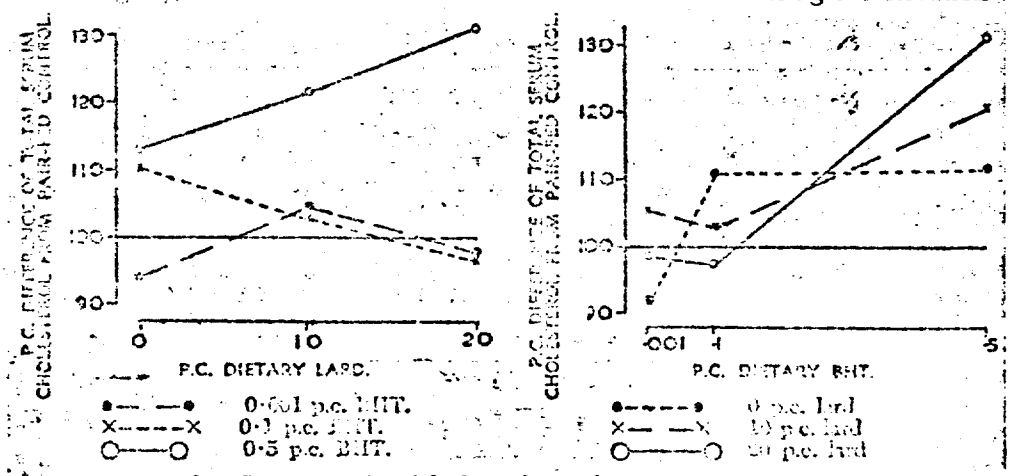


Fig. 3. The effect of BHT and lard on the total serum cholesterol levels of male rats expressed as a percentage of the serum cholesterol of paired controls.

of the serum cholesterol levels produced by 0·5 p.c. BHT. Increasing the lard content of a diet containing 0·1 p.c. BHT, antagonises the trend towards an increase in serum cholesterol produced in a non-lard-containing diet. A similar interaction between the lard and the BHT level of the diet on the serum phospholipid levels was noted (Table 3).

TABLE 5.

*The effect of BHT on the mean levels of some constituents of the male rat sera.*

| Determination, mean of 13 animals (mg./100ml.) | Percentage BHT content of diet (dry wght.) | | | | | | S.E.M. between paired treatment and control means on 20 d.f. |
|---|---|---|---|---|---|---|---|
| | 0 | 0·001 | 0·1 | 0 | 0·5 | 0 | |
| Fatty Acids | 193 | 190 | 191 | 190 | 208 | 193 | 16·1 |
| Mucoprotein | 164 | 155 | 167 | 164 | 170 | 163 | 7·5 |
| Hexosamine | 125 | 122 | 129 | 124 | 125 | 123 | 4·8 |

TABLE 6.

*The effect of chronic BHT on the mean levels of some constituents of the female rat sera.*

| Determination (mg./100 ml) | Number of animals | Percentage BHT content of diet (dry weight) | | | S.E.M. on 4 degrees of freedom |
|---|---|---|---|---|---|
| | | 0 | 0·1 | 0·5 | |
| Cholesterol (combined 10 p.c. and 20 p.c. lard supplements) | 16 | 99 | 100 | 122($p<0·001$[b]) | 2·5 |
| Cholesterol (10 p.c. lard supplement) | 8 | 93 | 109($p<0·05$) | 124($p<0·01$) | 3·6 |
| Cholesterol (20 p.c. lard supplement) | 8 | 99 | 92 | 123($p<0·01$) | 3·6 |
| Phospholipid (combined 10 p.c. and 20 p.c. lard supplement) | 16 | 156 | 163 | 124($p<0·05$) | 4·8 |
| Mucoprotein (combined 10 p.c. and 20 p.c. lard supplement) | 16 | 131 | 132 | 160($p<0·01$) | 6·7 |
| Fatty Acids (combined 10 p.c. and 20 p.c. lard supplement) | 16 | 160 | 169 | 157 | 3·6 |
| Hexosamine (combined 10 p.c. and 20 p.c. lard supplement) | | 149 | 147 | 161 | |

(a) Insufficient material for statistical analysis.

(b) Probability of significant difference from appropriate control.

No effects of BHT on the serum content of mucoprotein, hexosamine or total extracted fatty acids were observed (Table 5).

*Chronic study.*

Further confirmation of the relationship between the level of dietary fat, as a factor in the elevation of serum cholesterol produced by BHT, were observed in the chronic experiment (Table 6). A diet containing 0·1 p.c. BHT and 10 p.c. lard produced significant increases in the serum cholesterol of female rats. At this level of BHT with 20 p.c. lard supplement no increase was observed. A dietary level of 0·5 p.c. BHT produced significant increases in the cholesterol, phospholipid and mucoprotein content of female rat sera. No significant variations in the fatty acid content of the sera were noted on any of the dietary regimens. As insufficient quantities of sera were available for the estimation of the serum hexosamine in each instance, the mean values obtained could not be statistically analysed. However, this component appeared to increase with increased BHT content of the diet, this being consistent with the mucoprotein changes.

## DISCUSSION.

The elevations of the serum cholesterol and phospholipid levels observed show that the metabolism of relatively large doses of ingested BHT directly influences the serum levels of these constituents.

That 0·1 p.c. BHT, with a 10 p.c. lard supplement to the diet, produces an increase of serum cholesterol in female rats is of particular interest, as this dose level is referred to as the Normal Testing Dose (NTD) for a food additive. The NTD is that dietary level of a substance which is 100 times the maximum normally expected to be present in foodstuffs for human consumption (Lehman, 1951). One of the major criteria in the determination of the safety of a food additive is that continuous ingestion of the NTD will not cause any departure from normal cellular metabolism.

There is a very close correlation between the levels of cholesterol and phospholipid in serum, and the C/P ratios are constant under a variety of circumstances (Duncl, 1957). Hence the increases in the phospholipid and cholesterol content of the sera in these experiments probably have a common explanation.

It has been shown that the essential fatty acids (EFA) are necessary for the normal transport and metabolism of cholesterol and phospholipid (Kelsey and Longenecker, 1941; Allin-Slater *et al.*, 1954; Peifer and Holman, 1955). Further clarification of the role of fatty acids in the control of the serum cholesterol level has come from the works of Hegsted *et al.* (1957), who have shown that the serum cholesterol level in rats on a cholesterol-cholic acid regimen is inversely related to the products obtained by multiplying the EFA content (linoleic and arachidonic) of the diet by total saturated fatty acid content, the remain-

ing unsaturated fatty acids having a reverse effect in that they increase the cholesterol content of the sera.

It has been established that there is a relationship between the tocopherols, the natural antioxidants and the fatty acids. Thus the abnormal oxidation of highly unsaturated fatty acids within the tissues can be prevented by the *in vivo* antioxidant action of the tocopherols (Dam, 1953), and Kummerow *et al.* (1949) have shown that the tocopherols are functional in the synthesis of oleic and saturated acids.

Whether or not the synthetic antioxidants are metabolically active is debatable. Several synthetic antioxidants can protect an animal from some manifestations of vitamin E deficiency (Dam, 1953; Singsen *et al.*, 1955; Draper *et al.*, 1953). Johnson *et al.* (1953a) have shown that ingested BHT causes a reduction in the iodine value of perirenal fat in the rat, while Kummerow *et al.* (1949) established a further relationship between dietary antioxidants and the composition of depot fat when they showed that the synthetic antioxidant, butylated hydroxy anisole, like the tocopherols, is functional in the synthesis of oleic and saturated acids.

The above evidence, together with the results of this present study, suggests a tentative hypothesis that relatively large doses of BHT interfere with the normal metabolism of the fatty acids, this abnormal metabolism resulting in increased total serum cholesterol and phospholipid levels.

The tocopherols do not have the same action as BHT as, in the rat maintained on normal diets unsupplemented with cholesterol, no changes in blood cholesterol levels can be produced by hypervitaminosis E (Mounier *et al.*, 1941; Marx *et al.*, 1949; Aftergood *et al.*, 1957; Wissler *et al.*, 1953). BHT and three other synthetic antioxidants, when fed at a level of 0·025 p.c. in the diet, did not prevent the increase in plasma cholesterol and muscle lipid which occur before the onset of muscular dystrophy, induced in guinea-pigs by a vitamin E deficient diet. Supplements of α-tocopherol, fully protective against the occurrence of dystrophy, resulted in much lower levels in these lipid components (Shull *et al.*, 1953). In humans Gray and Loh (1955) have shown that ingestion of α-tocopherol produced increases in serum cholesterol, phospholipid, and the $\alpha_2$ globulin fraction, but has no effect on the fatty acid content. BHT produces very similar effects in the rat, as it is probable that these $\alpha_2$ globulin increases are analogous with the mucoprotein increases shown in the present study, especially as Bjornesjö (1955) has shown that the $\alpha_2$ globulin fraction contains the greatest percentage of polysaccharides.

The increase in mucoprotein material upon ingestion of BHT suggests a biochemical connection between BHT and the synthesis or degradation of ground substance mucopolysaccharides in the body. Alternatively, such changes in mucoprotein metabolism may reflect ground substance changes, secondary to altered fatty acid metabolism.

Johnson and O'Halloran (1958b) have shown that there is an increase in the liver size of rats fed the NTD of BHT, which was not accompanied by any pathological or histopathological changes. They suggest that the liver is the site of detoxication of BHT, the end products of which include glucuronides and ethereal sulphates (Dacre, 1957; Deichmann *et al.*, 1955), the increase in liver size being a response on the part of this organ to the stresses placed upon it by the detoxication of an abnormal exogenous material. If the detoxication capacity of the liver should be inadequate to deal with the relatively large doses of ingested BHT, this substance or its metabolites may accumulate in the blood or tissues and be responsible for the effects reported in this study. The lack of an elevation in serum cholesterol and phospholipid at lower levels of dietary BHT, reported here, and those of Shull *et al.* (1958) using vitamin E deficient guinea-pigs, may be attributed to the relative ease of hepatic detoxication of BHT.

It is difficult to interpret the effects found in the paired feeding experiment, resulting from different dietary supplements of lard and restricted food intake on the serum cholesterol, phospholipid and hexosamine levels of the control animals. In this study there was no attempt to substitute isocalorifically the lard for some other component of the diet. Supplementation of the diet by lard, with a subsequent reduction in the percentage of other components, did not lead to any significant variations in the body weight or renal and hepatic histopathology of the animals (Johnson and O'Halloran, 1958b). The significant reduction of the serum cholesterol levels on supplementation of the diet by lard, a relatively saturated fat, is not in accord with observations on humans reported from many different laboratories, which have shown that in general the serum cholesterol level is inversely related to the iodine value of the dietary fat. Our work together with that of Rose and Subrahmanyan (1950) and Best *et al.* (1958), who could show no significant differences in the total serum cholesterol of rats fed different fats of varying iodine values, suggests that the rat differs markedly from man and other species in the response of serum cholesterol to variations in the amount and degree of unsaturation of the dietary fat.

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# TOXICITY OF DITERTIARYBUTYLMETHYLPHENOL

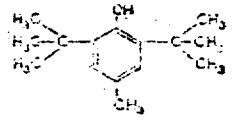
WILLIAM B. DEICHMANN, Ph.D., Coral Gables, Fla.
J. J. CLEMMER, M.D.
R. RAKOCZY, Dip.Ing.Chem.
and
J. BIANCHINE, A.B., Albany, N. Y.

DITERTIARYBUTYLMETHYLPHENOL is an antioxidant which in small amounts prevents the deterioration of a variety of materials, including fats, oils, waxes, resins, and plastic films. The present investigations are concerned with establishing safe limits for its use in foods and in materials entering into food packaging. To mention a few of the potential uses, it is expected that this material will ultimately be incorporated into most edible vegetable or animal fats and oils, into baked and fried foods, and into waxes or plastic films used for coating food wrappers or containers.

The data presented here define the immediate, subacute, and chronic toxicity of ditertiarybutylmethylphenol for experimental animals. These data can be used as a basis for estimating harmless levels of this material in food for human consumption.

## MATERIALS AND METHODS

Ditertiarybutylmethylphenol (DBMP) has the following formula:



It is known also as 4-methyl-2,6-ditertiarybutylphenol, as 2,6-ditertiarybutylparacresol, and as ditertiarybutylhydroxytoluene.* It is a very slightly yellowish crystalline solid which melts at 70C. It boils at 265C.

The studies were conducted with samples of the purified commercial product. Unless stated otherwise, the immediate and subacute toxicity tests were conducted with one commercial product.† The immediate toxicity was expressed in terms of an approximate lethal dose.[1] An $L.D._{50}$ was calculated from the experimental data employing the method of Miller and Tainter.[4] For the chronic tests, equal quantities of the above commercial product and another commercial product ‡ were mixed in the laboratory of the University and used as such. DBMP was received in wide-mouth dark bottles, which were filled and sealed in an atmosphere of nitrogen. The compound was stored in the dark until used.

The experimental animals employed included albino rats of Wistar stock, New Zealand rabbits, guinea pigs, cats, and mongrel dogs. The rats, rabbits, and guinea pigs were purchased from a local breeder. Generally, they were kept in isolation for two weeks before being employed. The cats and dogs were kept in the laboratory from four to six weeks prior to experimentation in order to ascertain their state of health. During this period the dogs were dewormed and protected against distemper.

This study was supported by a joint grant from Enjay Co., Inc., Koppers Co., Inc., and Shell Chemical Corp.

From the Department of Pharmacology, University of Miami School of Medicine (Dr. Deichmann) and the Departments of Pharmacology (Messrs. Rakoczy and Bianchine) and Pathology (Dr. Clemmer), Albany Medical College.

* Available commercially under the trade names of Deenax or Paranox 441 (Enjay Co., Inc.), DBPC Antioxidant (Koppers Co., Inc.), and Ionol (Shell Chemical Corp.).

† Supplied through the courtesy of Mr. N. L. Gianakos, of Shell Chemical Corp. By private communication, this material is reported to have a melting point ranging from 69.3 to 69.7 C.

‡ Supplied through the courtesy of Dr. Gilbert Thiessen, of the Koppers Co., Inc. By private communication, this material is reported to have a melting point of 68.5 C. which corresponds to a mole purity of 97.8%, based on the freezing point of pure DBMP of 69.82 C.

All animals were kept supplied with food and water at all times. The rats and dogs used for the determination of the immediate and subacute toxicity studies were fed whole or ground Purina dog pellets. Rats and dogs subjected to the one- and two-year studies were fed Big Red Dog Meal (Coop.GLF Service). In addition, the dogs were given at intervals ground meat or Laddie Boy Horse Meat. The guinea pigs and rabbits were supplied with Purina rabbit pellets. The cats were fed Puss-N-Boots cat food plus milk.

DBMP was incorporated in a diet as a solution in liquefied lard; this was obtained at intervals from Swift & Company, of Chicago, and was guaranteed to be free of antioxidants.

The method of one of us (W. B. D.) was employed for the estimation of glucuronic acid in urine.[2] The procedure employs the naphthoresorcinol reagent and is therefore a modification of Tollen's qualitative test. A fair degree of accuracy is claimed for this method, in spite of the nonspecificity of the reagent. The ratio of inorganic to organic sulfates was determined by the method of Treon and Crutchfield.[5]

Two methods were used to determine whether or not DBMP will produce hypersensitivity. The first procedure is a modification of that described by Draize.[8] To carry out this test, the hair was clipped very short on the back and flanks of each of five guinea pigs. On three days of a week each animal was given an intracutaneous injection employing a 26 gauge hypodermic needle. On the first day, 0.04 mg. of DBMP in 0.05 ml. of 10% aqueous ethyl alcohol was injected. Subsequent injections were equal to 0.08 mg. of DBMP in 0.1 ml. of 10% alcohol, until a total of 10 doses had been administered. Two weeks after the administration of the 10th dose, 0.04 mg. of DBMP was injected and its effect observed and recorded on the following day. The second method was designed to produce anaphylactic shock in a guinea pig sensitized with DBMP. A sensitizing dose of 0.08 mg. of DBMP in 0.1 ml. of 10% aqueous alcohol was injected intraperitoneally. Two weeks later a shock dose of 0.16 mg. in 0.2 ml. of 10% alcohol was administered. All animals subjected to an acute experiment were kept under observation for a period of three weeks; many were observed for four weeks.

## RESULTS

### Immediate Toxicity

When administered at one time by stomach tube to rats, rabbits, guinea pigs, and cats toxic doses of DBMP (20% solution in corn oil) induced salivation, a mild degree of miosis, restlessness, hyperexcitability, unsteadiness, increased urination, diarrhea, tremors, and a more or less pronounced degree of paralysis of the hindquarters. Fatal doses generally induced for days complete loss of appetite and finally respiratory difficulties and coma. The feces of the animals given large oral doses of DBMP often showed the presence of blood. After oral administration of one approximate lethal dose or $L.D._{50}$ most of the rats, cats, and guinea pigs survived for 2 to 3 days; rabbits, however, survived for periods ranging from 4 to 26 days (Table 1).

Table 1.—*Immediate Toxicity of Ditertiarybutylmethylphenol*

(The compound was administered as a 20% solution [W/V] in corn oil; after treatment, animals were observed for periods ranging from two to four weeks; an approximately equal number of males and females were used.)

| Species | Animals Treated, No. | Periods of Survival | $L.D._{50}$, Gm./Kg. | Approx. Lethal Dose, Gm./Kg. |
|---|---|---|---|---|
| Cat | 18 | 38 hr. to 5 days | .... | 0.94 to 2.1 |
| Rat (male) | 42 | 2 to 7 days | (3.7) | ..... |
| Rat (female) | 42 | 2 to 3 days | 3.87 | ..... |
| Rabbit | 12 | 4 to 26 days | .... | 2.1 to 3.2 |
| Guinea pig | 7 | 4 days | .... | 10.7 |

### Subacute Toxicity

*Twenty-Eight-Day Feeding Experiment (Dogs).*—DBMP was incorporated in ground meat and fed to each of four dogs for a period of four weeks. During the first two weeks the animals were offered two doses per week and during the last two

weeks three doses per week, each dose equal to 1.4, 2.1, 3.2, or 4.7 gm. per kilogram, respectively. The total quantities of DBMP ingested during the four-week period were equal to 93, 102, 233, and 488 gm. per dog, respectively. On the basis of grams per kilogram per dog, these quantities are equivalent to 11.2, 8.4, 19.2, and 37.6.

During the first week of the feeding experiment each of the four animals responded with a mild to a moderate degree of diarrhea. This became apparent in one to three hours after ingestion of DBMP. During the second and third week the onset of diarrhea became more and more delayed. During the fourth week an animal, regardless of the dose, showed only a very mild degree of diarrhea, with an onset approximately 24 hours after the ingestion of DBMP and lasting for about one day. No other signs of intoxication were observed.

At the termination of the four-week experiment, all animals were killed. Gross pathological tissue changes were absent, with the exception of some hemorrhagic areas, edema, and congestion of the lungs. It is believed that these changes were caused by the mode of killing, i. e., air embolism. There was no sign of irritation in the gastroenteric tract.

*Fourteen-Day Feeding Experiment (Rabbits) and Twenty-Day Feeding Experiment (Rats).*—DBMP was incorporated in ground feed (15 rats, weighing 120 to 148 gm.) and in pellets (12 rabbits, weighing 1.2 to 1.4 kg.) to an extent of 1 and 5%. Both concentrations were fed for a period of 20 days in the case of rats and for 14 days in the case of rabbits. All experimental animals lost weight (rats, 2 to 41 gm.; rabbits, 220 to 370 gm.) with the exception of one rabbit. This loss is believed to have been due primarily to a subnormal intake of contaminated food. The daily food consumption was not recorded. One rabbit which was fed a diet containing 5% DBMP died 10 days after discontinuation of the feeding experiment, while one of three rats fed 5% DBMP died 8 days after initiation of this study.

*Ninety-Day Feeding Experiment (Rats).*—Two grades of DBMP were added to the feed. They differed very slightly in degree of purity and represented "DBMP, M.P. 69.7 C., purity 99.7% wt." and "DBMP, M.P. 69.3 C., purity greater than 98.8% wt." Each of these materials was dissolved and incorporated in ground feed to obtain concentrations of 0.2, 0.5, 0.8, and 1.0% DBMP, respectively, plus 1.0% of lard. Four groups of rats were fed "DBMP, M.P. 69.7 C." and four groups received the other material. In addition, certain control groups were provided. One received ground feed containing 1.0% lard; the other was given an uncontaminated control diet. Two additional groups of rats were started on a diet containing 0.2% DBMP plus 1.0% lard, but in this experiment the concentration of DBMP was increased every three or four days until a concentration of 1.5% was reached. There were three rats in each of the groups.

There was no significant difference in the effects noted after feeding the two grades of DBMP. Contamination of the diet with 0.2% of DBMP induced no signs of intoxication. Of the two groups fed 0.5%, one of six rats died after 12 days; feeding of 0.8% resulted in two deaths (4 and 5 weeks), and feeding of 1.0% caused the death of one rat 13 weeks after initiation of the 90-day feeding experiment. Rats which were fed gradually increasing concentrations of DBMP refused contaminated feed almost entirely when concentrations reached 1.5%. Four of six rats died (4, 4, 9, and 11 weeks).

*Paired Feeding Experiment.*—There were 5 male rats in each of the control and the experimental groups fed a diet containing 0.8% DBMP, but there were 10 male rats in the groups fed a diet containing 1.0% DBMP. All animals were housed in groups of five. Each day the weight of the contaminated food ingested was recorded. This same quantity, in the form of uncontaminated food, was fed the following day to the respective control group. Rats of an experimental and a control group consumed therefore the same quantity of food. The experiment was carried out for a period of 25 days.

Preliminary feeding experiments had shown that rats and rabbits refuse food which contains 1.0% or a higher concentration of DBMP. Earlier feeding experiments had also demonstrated that rats will accept food contaminated with 1.0% of DBMP as long as this concentration is reached gradually. In the paired feeding experiments, to be reported here, rats were placed on a diet containing 0.4% DBMP. On the fourth day the concentration of DBMP was increased to 0.8%, and on the seventh day to 1.0%. Each experiment was carried out for a period of 25 days. From these we learn the following facts:

1. Control male rats: fed an uncontaminated diet ad lib.
   - Mean daily food intake.... 14.7 gm.
   - Mean total gain of body weight ..... 129.0 gm.
2. Paired male rats: fed a diet contaminated to an extent of 0.8% DBMP
   - Mean daily food intake.... 12.3 gm.
   - Mean total gain of body weight
     - (*a*) Experimental group. 78.0 gm.
     - (*b*) Control group..... 65.0 gm.
3. Paired male rats: fed a diet contaminated to an extent of 1.0% DBMP
   - Mean daily food intake.... 12.8 gm.
   - Mean total gain of body weight
     - (*a*) Experimental group. 28.0 gm.
     - (*b*) Control group..... 65.0 gm.

It becomes apparent that (*a*) a diet containing 0.8% of DBMP produces anorexia or is unpalatable to rats and (*b*) feeding of a diet containing 1.0% DBMP will retard growth.

## Chronic Toxicity

*One-Year Feeding Experiment Employing Dogs.*—Six mongrel dogs (weight 5.5 to 16.2 kg.) were employed. An attempt was made to feed each of two dogs a daily dose of 0.25, 0.50, or 1.0 gm. of DBMP per kilogram, respectively. The material was mixed with a small quantity of ground meat. After ingestion of this mixture, a dog was fed a ration of Dog Meal. Occasionally each dog refused part or all of the contaminated meat, and an estimate was then made of the quantities ingested. The daily quantity of the antioxidant consumed was recorded, and from these data, plus the weight of the dog (determined once per week), the animal's mean weekly (five-day period) intake and finally the mean annual ingestion of DBMP were calculated. Most of the dogs were nearly full-grown animals; obviously, only a moderate increase in body weight was expected and observed.

For a period of 12 months (on each of five days of a week) each of six dogs ingested from 0.17 to 0.94 gm. of DBMP per kilogram (Table 2). The total quantities consumed ranged from 483 to 2,402 gm. per dog per year, or from 45 to 244 gm. per kilogram per dog per year.

Table 2.—*Twelve-Month DBMP Feeding Experiment Employing Six Dogs*

| Dog, Identification No. | Dose of DBMP Offered Dog on Each of Five Days per Week, Gm./Kg. | Average Dose of DBMP Ingested on Each of 260 Days Over Period of One Year, Gm./Kg. | Total Quantity of DBMP Ingested Over Period of One Year | |
|---|---|---|---|---|
| | | | Gm./Dog | Gm./Kg. |
| D-7066 (52) | 0.25 | 0.17 | 483.3 | 45.88 |
| D-7065 (53) | 0.25 | 0.23 | 722.2 | 60.58 |
| D-7453 (54) | 0.50 | 0.50 | 2,220.6 | 132.10 |
| D-7155 (55) | 0.50 | 0.47 | 2,002.0 | 122.37 |
| D-7067 (56) | 1.00 | 0.64 | 1,151.2 | 166.36 |
| D-7064 (57) | 1.00 | 0.94 | 2,402.2 | 244.11 |

No dog displayed signs of intoxication during the course of this experiment. The compound did not produce diarrhea, as was noted after ingestion of higher daily doses (3 to 37 gm. per kilogram per dog per 28 days); there was no excretion of blood with the feces. Upon completion of the 12-month feeding period, five of the six dogs were killed by electrocution, and their organs and tissues were examined. One animal became pregnant and delivered seven normal puppies 11 months after initiation of experimental feeding. This dog was killed two months later.

Histopathological studies were carried out on the following organs: brain, pituitary, thyroid, thymus, heart, aorta, trachea, lungs, spleen, stomach, intestine, pancreas, liver, mesenteric lymph nodes, adrenal, kidney,

urinary bladder, uterus, ovary, testis, epididymis, and prostate.

No gross or microscopic pathology was observed in any of the six dogs studied, which can be attributed to the feeding of DBMP. The only pathology was in Dog 55, in the form of lobular pneumonia and focal necrosis of the liver. This pathology was considered to be the result of a recent infection and not attributal to the feeding of DBMP.

*Two-Year Feeding Experiment Employing Rats.*—Separate groups of male and female rats were placed on diets containing 0.2, 0.5, 0.8, or 1.0% of DBMP in 1.0% of lard, along with a control group which was fed a diet containing 1.0% lard without DBMP. It was considered that heating of the lard, as is done in "deep frying," might possibly affect the stability and toxicity of DBMP. To test this, an additional chronic experiment was conducted, in which, prior to incorporation in the diet, the DBMP was dissolved in lard and heated for 30 minutes at 150 C. The concentration of DBMP in food tested in this manner was equal to 0.5%. One group of rats was fed an uncontaminated diet but containing 1.0% of lard. There was therefore a total of six groups, with 15 female and 15 male rats per group. The females were housed in groups of seven or eight; the males, in groups of two or three. Contaminated feeds were prepared once a week or oftener and stored in a refrigerator until fed.

All animals were weighed in groups once a week, but when the weights became fairly constant, weighings were reduced to once a month. Blood counts and determinations for hemoglobin were carried out at intervals during this investigation. Rats selected for these blood tests were picked at random; if any selection was made, it was in an effort to take those that did not appear as healthy as others. At termination of the two-year feeding experiment, all surviving rats were killed by exposure to vapors of chloroform; they were subsequently examined for gross pathological changes. The weight of each of the larger organs was determined, and sections were taken and examined for micropathological changes.

The following observations were made:

1. Over the two-year period, the number of fatalities ranged from six to nine rats per group of females and from four to seven per group of males. There was no relation between the number of fatalities and the concentration of DBMP in the diet, including the control uncontaminated diet.

2. Feeding of DBMP had no specific effect on the number of erythrocytes and leucocytes or on the concentration of hemoglobin in the peripheral blood. A total of 277 blood specimens were examined.

3. Gain of body weight was normal, regardless of the concentration of DBMP in the diet, with the exception of the gain of weight of male and female rats fed 1.0% of DBMP. Ingestion of this dose resulted in a subnormal increase of weight (Tables 3 and 4).

4. Feeding of diets containing up to and including 0.8% of DBMP had no specific effect on the weights of organs (brain, heart, lungs, kidneys, spleen, liver) of rats that survived the 24-month feeding experiment. In the groups fed 1.0% DBMP there was, however, a tendency for an increased weight of certain organs in relation to an animal's body weight, involving brain, lungs, and liver in the case of the females, and brain, liver, and testes in the males (Tables 3 and 4).

5. Sections of the following organs were studied microscopically after their paraffin embedding and staining with hematoxylin and eosin: brain, heart, lungs, spleen, liver, kidneys, and enteric tract, including esophagus, stomach, and small and large intestine. Sections of liver and kidneys were prepared by the frozen section technique and stained with Sudan III for fat. In all, 353 sections stained with hematoxylin and eosin and 82 sections stained with Sudan III were examined.

Various types of pathology were observed, but the pathology followed no significant pattern. Lesions observed in the female rats fed DBMP, including even the group fed 1.0%

of this compound, were similar to those observed in the control females. Evidence of pneumonia was found in several of the rats. Four of the six animals in the control group exhibited malignant neoplasms (two, reticulum cell sarcomas; two, carcinomas of the breast). The female rats in the DBMP-fed groups included five with malignant neoplasms (three, reticulum cell sarcomas; one, carcinoma of the breast, and one leukemia). Many of the killed animals exhibited no demonstrable anatomic pathology.

The male animals also exhibited various types of pathology; again the pathology followed no significant pattern. Bronchitis and pneumonia were fairly frequent findings, but these changes were observed in the control group and more frequently in the groups fed a low per cent of DBMP than in the groups receiving the higher levels. One male rat showed evidence of leukemia, and in two rats in the control group neoplasms were found; one was a reticulum cell sarcoma of the lung and the other a fibroma in the mammary

TABLE 3.—*Twenty-Four Month DBMP Feeding Experiment Employing Fifteen Female Rats per Group*

| | Group 6 (Control) | Group 1 (0.2%) | Group 2 (0.5%) | Group 5 (0.5% Heat) | Group 3 (0.8%) | Group 4 (1.0%) |
|---|---|---|---|---|---|---|
| **Effect on Body Weight** | | | | | | |
| Gain of body weight over 24 months | 237 gm. | 224 gm. | 241 gm. | 243 gm. | 228 gm. | 172 gm. |
| **Effect on Hemopoietic System** | | | | | | |
| Erythrocytes (millions/cu. mm.) | | | | | | |
| Mean | 8.2 | 8.2 | 8.7 | 8.1 | 8.9 | 9.6 |
| 90% values | 6.0 to 9.2 | 6.8 to 9.9 | 7.2 to 10.5 | 6.9 to 9.1 | 7.6 to 10.8 | 7.9 to 11.2 |
| Leucocytes (thousands/cu. mm.) | | | | | | |
| Mean | 13.4 | 9.6 | 10.1 | 10.8 | 10.4 | 9.9 |
| 90% values | 7.7 to 22.6 | 5.4 to 18.8 | 7.0 to 15.8 | 5.3 to 15.1 | 6.2 to 17.5 | 6.0 to 16.0 |
| Hemoglobin (gm./100 ml.) | | | | | | |
| Mean | 14.5 | 14.1 | 14.4 | 15.0 | 14.6 | 14.3 |
| 90% values | 12.2 to 16.8 | 13.0 to 15.1 | 12.8 to 15.3 | 13.4 to 16.1 | 12.3 to 16.8 | 13.0 to 15.5 |
| **Organ Weight as Per Cent of Body Weight** | | | | | | |
| Brain | 0.618 | 0.681 | 0.630 | 0.606 | 0.668 | 0.765 |
| Heart | 0.480 | 0.531 | 0.475 | 0.469 | [illegible] | 0.551 |
| Lungs | 0.996 | 0.741 | 0.768 | 1.04 | 1.02 | 1.20 |
| Kidneys | 0.910 | 1.01 | 0.854 | 0.920 | 0.778 | 0.807 |
| Spleen | 0.290 | 0.290 | 0.257 | 0.308 | 0.249 | 0.289 |
| Liver | 4.57 | 4.25 | 4.10 | 5.20 | 5.19 | 5.32 |

TABLE 4.—*Twenty-Four-Month DBMP Feeding Experiment Employing Fifteen Male Rats per Group*

| | Group 6 (Control) | Group 1 (0.2%) | Group 2 (0.5%) | Group 5 (0.5% Heat) | Group 3 (0.8%) | Group 4 (1.0%) |
|---|---|---|---|---|---|---|
| **Effect on Body Weight** | | | | | | |
| Gain of body weight over 24 months | 367 gm. | 362 gm. | 352 gm. | 389 gm. | 324 gm. | 241 gm. |
| **Effect on Hemopoietic System** | | | | | | |
| Erythrocytes (millions/cu. mm.) | | | | | | |
| Mean | 8.9 | 9.2 | 9.8 | 9.0 | 9.4 | .... |
| 90% values | 7.3 to 10.4 | 7.0 to 12.1 | 6.7 to 13.7 | 6.8 to 12.6 | 6.9 to 12.0 | .... |
| Leucocytes (thousands/cu. mm.) | | | | | | |
| Mean | 16.7 | 16.4 | 12.3 | 15.8 | 16.4 | .... |
| 90% values | 5.5 to 29.7 | 7.6 to 32.5 | 5.6 to 19.7 | 9.4 to 26.9 | 8.2 to 33.0 | .... |
| Hemoglobin (gm./100 ml.) | | | | | | |
| Mean | 14.8 | 14.6 | 14.5 | 14.5 | 14.5 | .... |
| 90% values | 13.8 to 16.4 | 13.6 to 16.2 | 13.6 to 15.5 | 13.2 to 15.7 | 12.7 to 16.0 | .... |
| **Organ Weight as Per Cent of Body Weight** | | | | | | |
| Brain | 0.476 | 0.481 | 0.483 | 0.478 | 0.527 | 0.663 |
| Heart | 0.397 | 0.387 | 0.386 | 0.339 | 0.331 | 0.306 |
| Lungs | 1.12 | 1.34 | 1.00 | 1.04 | 0.781 | 0.874 |
| Kidneys | 0.839 | 0.884 | 0.968 | 0.864 | 0.803 | 0.897 |
| Spleen | 0.402 | 0.278 | 0.376 | 0.303 | 0.291 | 0.316 |
| Liver | 3.85 | 4.04 | 4.55 | 4.51 | 4.87 | 5.85 |
| Testes | 0.774 | 0.804 | 0.758 | 0.800 | 0.862 | 1.01 |

TABLE 5.—*Effect of DBMP on Excretion of Glucuronic Acid in Urine of the Rabbit*

| Date | Total Vol. of Urine in 24 Hr., Ml. | Glucuronic Acid in Urine: Mg./24 Hr. Sample | Glucuronic Acid in Urine: Mg./100 Ml. |
|---|---|---|---|
| Each of two animals given one dose of 3.2 gm. of DBMP per kilogram as a 5% solution in corn oil; a composite specimen collected over a 24-hour period. | | | |
| 6/ 8 (control) | 165 | 58.8 | 54.4 |
| 6/ 9 (control) | 79 | 51.9 | 65.7 |
| 6/10 | 292 | 530.8 | 205.7 |
| 6/11 | 120 | 117.2 | 97.7 |
| 6/13 | 285 | 994.7 | 349.0 |
| 6/14 | 234 | 234.6 | 100.2 |
| 6/15 | 75 | 71.5 | 195.3 |
| 6/16 | 225 | 71.1 | 31.6 |
| Each of two animals given six oral doses of 0.3 gm. of DBMP per kilogram (each dose approximately 12% of approximate lethal dose) as a 5% solution in corn oil over a period of seven days; a composite specimen collected over each 24-hour period. | | | |
| 6/14 (control) | 165 | 99.4 | 60.5 |
| 6/15 | 150 | 141.2 | 94.1 |
| 6/16 | 150 | 122.0 | 82.5 |
| 6/17 | 160 | 107.5 | 67.1 |
| 6/18 | 110 | 63.7 | 57.9 |
| 6/20 | 110 | 92.5 | 84.1 |
| 6/21 | 50 | 59.0 | 118.0 |

region. Many of the killed rats showed no demonstrable pathology. This was true of all of those examined from the group which was fed 1.0% DBMP.

It is concluded that DBMP administered to female and male rats in the manner described in this experiment produces no anatomic pathology.

## EFFECTS OF DBMP

*Effect of DBMP on Excretion of Glucuronates and Sulfates in Urine of Rabbits.*—DBMP induced in rabbits a slight to a moderately increased excretion of glucuronates in urine. The quantities excreted were in relation to the doses administered (Table 5). After absorption of DBMP, the ratio of inorganic to total sulfates decreased. These studies indicate that DBMP is probably detoxified in part by conjugation with glucuronic and sulfuric acids (Table 6).

*Effect of DBMP on Coagulation and Prothrombin Times.*—When administered to rabbits, toxic doses of DBMP did not influence significantly the clotting and prothrombin times of cardiac blood.

*Effect of DBMP on Production of Hypersensitivity in the Guinea Pig.*—DBMP did not induce a hypersensitive state in the guinea pig; nor did a guinea pig given a shock dose of DBMP (two weeks after injection of a sensitizing dose) demonstrate signs of anaphylaxis. When injected intracutaneously, DBMP induced only signs of local irritation and necrosis. All guinea pigs employed for these tests (conducted by A. Swift) gained weight and appeared healthy.

*Effect of DBMP on Blood Pressure.*—Conventional methods were employed to determine the effect of DBMP on blood pressure in the carotid artery of the dog. Pentobarbital (25 mg. per kilogram I.V.) was employed as an anesthetic.

The intravenous injection of 25 mg. of DBMP per kilogram of body weight (lecithin emulsion, 20 mg. of DBMP per milliliter) was followed by a prompt reduction in blood pressure from 105 to 40 mm. Hg. The pressure remained at this level for about 3 minutes, then rose over the following 15 minutes to 93 mm. Hg. In other dogs, the injection of 15 mg. of DBMP per kilogram induced a drop of pressure from 102 to 80 mm. Hg, while a dose of 40 mg. per kilogram was followed by a prompt reduction in pressure from 95 to 32 mm. Hg; this was followed

TABLE 6.—*Effect of DBMP on Excretion of Sulfate in Urine of the Rabbit*

The compound was administered in one dose by stomach tube as a 10% solution in corn oil. In some instances, two rabbits were kept in one metabolism cage and a composite urine sample was analyzed. (The ratio of "inorganic" to "total" sulfates found in the urine of untreated rabbits ranged from 85 to 95%.)

| Rabbit, Identification No. | Dose of DBMP, Gm./Kg. | Total Vol. of Urine in 48 Hr., Ml. | Ratio of Inorganic to Total Sulfates, % |
|---|---|---|---|
| D-4886 | 0.6 | 60 | 46.8 * |
| D-142 | 0.6 | ... | .... |
| D-143 | 0.6 | 205 | 59.7 * |
| D-4887 | 1.2 | 200 | 85.7 * |
| D-144 | 2.0 | ... | .... |
| D-145 | 2.0 | 240 | 66.2 * |
| D-106 | 0.3 | ... | .... |
| D-107 | 0.3 | 200 | 81.0 |
| D-108 | 0.6 | ... | .... |
| D-109 | 0.6 | 246 | 49.8 |
| D-110 | 1.8 | ... | .... |
| D-111 | 1.8 | 194 | 79.3 |
| D-112 | 3.2 | ... | .... |
| D-113 | 3.2 | 312 | 56.2 |

* These analyses were carried out by Dr. J. Treon, of the Kettering Laboratory, University of Cincinnati.

by recovery (to 86 mm. Hg) over a period of 40 minutes.

Pretreatment of a dog with atropine sulfate (0.3 mg. per kilogram I.V.) offered a mild degree of protection. After atropine, 25 mg. of DBMP per kilogram induced a drop of blood pressure from 103 to 82 mm. Hg.

It is concluded that the vasodepressor effect of DBMP is partially mediated through stimulation of parasympathetic innervations.

## CONCLUSIONS

1. When absorbed in toxic concentrations into the tissues of unanesthetized animals, ditertiarybutylmethylphenol (DBMP) induced signs of intoxication resembling those seen after absorption of a toxic dose of a parasympathetic drug (salivation, a mild degree of miosis, unsteadiness, restlessness, hyperexcitability, diarrhea, tremors). When given intravenously to a dog under pentobarbital anesthesia, DBMP (25 mg. per kilogram) induced a prompt reduction of blood pressure. Atropine sulfate partially antagonized this depressor effect.

2. A mild to a moderately severe degree of diarrhea was induced in dogs fed daily doses of 1.4 to 4.7 gm. of DBMP per kilogram in the diet over a period of four weeks. This amounted to a total consumption of quantities ranging from 93 to 488 gm. per dog, or 8 to 37 mg. per kilogram of dog weight. Postmortem examinations revealed no significant gross pathological changes.

3. No signs of intoxication or evidence of gross or histopathological changes were observed in dogs fed daily doses of 0.17 to 0.94 gm. of DBMP per kilogram in the diet over a period of 12 months. This amounted to a total consumption of quantities ranging from 483 to 2,402 gm. per dog or 45 to 244 gm. per kilogram of dog weight.

4. Feeding experiments conducted over periods of 20 and 90 days, respectively, indicate that rats do not find food palatable containing 0.5 or 1.0% of DBMP. The animals will, however, ingest foods so contaminated more readily if these concentrations in the diet are attained gradually. DBMP was incorporated in all diets fed rats as a solution in liquified lard guaranteed by Swift and Company to be free of antioxidants.

5. Paired feeding experiments (rats) demonstrated that the feeding of diets containing 0.8 and 1.0% of DBMP will reduce the daily intake of food below control values; a level of 1.0% of DBMP in the diet retarded the gain of body weight.

6. Male and female rats placed for a period of 24 months on diets contaminated with DBMP to the extent of 0.2, 0.5, and 0.8%, respectively, suffered no specific signs of intoxication and no anatomic pathology. An additional 0.5% feeding experiment was conducted. In this, DBMP, after it was dissolved in lard, was heated for 30 minutes at 150 C. and then incorporated in the diet. Again, no specific effects or micropathology were noted. The feeding of 1.0% of DBMP was followed in both male and female rats by a subnormal gain and by an increased weight of certain organs in relation to body weight. Anatomic pathology was also negative in this group.

A number of male and female rats died during the course of this experiment, but since the fatalities per group (including the control group) were in no relation to the concentration of DBMP fed, it is believed that the causes of death were unrelated to the feeding of this substance. The micropathological studies support this contention.

DBMP in the concentrations fed has no specific effect on the number of erythrocytes and leucocytes or on the concentration of hemoglobin in the peripheral blood of male and female rats.

7. DBMP is capable of inducing from a slight to a moderately increased excretion of glucuronates and organic sulfates in the urine of rabbits.

8. Toxic doses of DBMP did not influence coagulation and prothrombin times in the rabbit.

9. It was not possible to induce with DBMP a state of hypersensitivity in the guinea pig.

10. It can be concluded from these studies that ditertiarybutylmethylphenol is a rela-

tively innocuous compound. The fact that no toxic effects were observed with 0.8% DBMP in the diet, but slight retardation of weight gain was observed at the 1% level, indicates a relatively sharp break-point in the dose-response curve. Other materials which have such a sharp break-point in the dose-response curve exhibit no hazard when used at levels well below this break-point. Since the concentrations suitable for antioxidant purposes are well below the above levels, it is anticipated that ditertiarybutylmethylphenol could be used as an antioxidant in edible foods and in food wrappers or containers without any public health hazard.

Swift and Company, of Chicago, regularly supplied lard free of antioxidants for use in this study.

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# Liver Response Tests. I. Exploratory Studies on Glucose 6-phosphatase and Other Liver Enzymes

G. FEUER, L. GOLBERG and J. R. LE PELLEY

*British Industrial Biological Research Association, Woodmansterne Road, Carshalton, Surrey, England*

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Abstract—Experiments have been directed towards the detection of changes in the activity of some enzyme systems in the liver cell of rats given various compounds.

The following liver enzymes have been studied:- mitochondria. succinic de hydrogenase; microsomes: glucose 6-phosphatase, azo reductase; lysosomes: acid phosphatase, β-glucuronidase, cathepsin, arylsulphatase; cytoplasm: glucose 6-phosphate dehydrogenase.

Untreated rats have been compared with rats treated with hepatotoxic compounds (carbon tetrachloride, safrole, allyl alcohol, coumarin, β-thujone, ethionine, thioacetamide, 3-methyl-4'-dimethylaminoazobenzene, dimethylnitrosamine, aflatoxin); food additives (butylated hydroxyanisole, butylated hydroxytoluene, sucroglyceride); stabilizers (dioctyltin, 2-phenylindole); drugs (Drazine, sodium barbitone) and other compounds (Triton X-100, dimethylsulphoxide).

A significant reduction in glucose 6-phosphatase activity was observed when any of the hepatotoxic compounds were administered in appropriate dose levels and for appropriate lengths of time. Similarly, 2-phenylindole and butylated hydroxytoluene when given in high doses brought about a decrease in the activity of this enzyme. The other compounds did not elicit any significant reduction of glucose 6-phosphatase.

Glucose 6-phosphate dehydrogenase activity was increased following treatment of rats with carbon tetrachloride, coumarin, ethionine, dimethylnitrosamine, 2-phenylindole and high doses of butylated hydroxytoluene.

Changes in the activity of the lysosomal enzymes tested did not run parallel. After the administration of hepatotoxic compounds acid phosphatase and arylsulphatase were increased, β-glucuronidase and cathepsin did not show marked change. Sodium barbitone treatment did not result in any change of the lysosomal enzymes.

No difference was found in the activity of succinic dehydrogenase activity in the liver mitochondria of rats treated with various compounds.

## INTRODUCTION

From time immemorial, man's diet has contained a large variety of toxic substances. Experience has taught him to avoid the acute poisons but those of a more chronic and insidious character have probably remained largely undetected. To-day there is superimposed on this background a wide and increasing exposure to chemical substances, not only in food but also in the air, in water, at home and at work.

Liver injury is among the commonest manifestations of long-term toxic action. In testing new compounds, recognition of their hepatotoxic potential, particularly of low orders of toxicity, often presents considerable difficulties and uncertainties which can only be overcome by carrying out tests at high doses over long periods. The use of high doses brings in its train other problems, while the need for prolonged tests inevitably restricts technical development without necessarily increasing the assurance of safety from toxic hazard.

Biochemical tests for liver injury are usually carried out in the course of the study of short-term or long-term toxicity in animals. The tests applied are largely derived from procedures evolved for clinical assessment of liver function in ill patients. They are measurements of the degree of residual hepatic function and are needed for purposes of diagnosis,

prognosis and generally as guides to therapy. None of these objectives is of value in laboratory tests on healthy animals. The problem here is twofold: to detect adverse effects on the liver which are insufficient to produce frank histopathological changes; and, where an increase in relative liver weight is noted, to provide biochemical data that will facilitate the interpretation of this observation in terms of an adaptive change ('work hypertrophy') or of a manifestation of toxicity. The answer to this problem lies in developing biochemical measurements that will assess the liver's response to exposure to chemical agents. Such tests of liver response must reflect the functional state of those cytostructural elements that are believed on morphological grounds to be the most readily susceptible to damage. Above all, those tests will be of most value that point clearly and reliably to a pejorative influence on the liver at a stage when the extent of the damage is slight and not detectable by other means.

Although many studies have been conducted into the nature of the biochemical changes produced by toxic compounds (Mueller & Miller, 1950; Conney, Miller & Miller, 1956, 1957; Conney, Gillette, Inscoe, Trams & Posner, 1959; Fouts & Adamson, 1959; Pitot, 1960; Recknagel & Lombardi, 1961; Pitot, 1962; Wattenberg, Leong & Strand, 1962; Chang, Ward & Ichinoe, 1963; Heise & Görlich, 1964; Koransky, Portig, Vohland & Klempau, 1964; de Man, 1964), emphasis has almost always been placed on highly toxic materials. Insufficient attention has been paid to the detection of early changes induced by substances possessing a low toxic potential. Our interest has been especially directed towards the detection of slight alterations to the biochemical organization of the liver cell caused by diverse compounds possessing very different toxicities. By this means attempts have been made to recognize early manifestations of toxic damage. In most circumstances it is likely that disturbances in the function and dynamic equilibrium of various enzyme systems precede recognizable pathological changes. It seemed reasonable, therefore, to investigate enzyme changes which might be associated with hepatotoxic effects.

A preliminary account of this investigation has been published elsewhere (Feuer, Golberg & Le Pelley, 1964).

## EXPERIMENTAL

*Materials.* Carbon tetrachloride, allyl alcohol, safrole, dimethylnitrosamine, dimethylsulphoxide, DL-ethionine, thioacetamide, and sodium barbitone were obtained from British Drug House, Ltd., Poole, Dorset; AnalaR or Laboratory grade reagents were used. Dimethylnitrosamine was redistilled before use. Coumarin (100%) and $\beta$-thujone (pure 99·5%) were obtained from W. J. Bush and Co. Ltd., London. 3-Methyl-4′-dimethylaminoazobenzene was provided by Benger Laboratories Ltd., Holmes Chapel, Cheshire, and aflatoxin P41 (assay: 44% $B_1$, 44% $G_1$ and remainder $B_2$, $G_2$ and other impurities) prepared by Medical Research Council (MRC) Laboratories, Mill Hill, Middlesex, and kindly given by Dr. J. M. Barnes. Butylated hydroxytoluene and butylated hydroxyanisole were supplied by May & Baker Ltd., Dagenham, Essex; dioctyltin derivative (Stanclere 176) prepared by Pure Chemicals Ltd., Liverpool, Lancashire; palm oil sucroglyceride was obtained from Lofil Ltd., London; Triton X-100 was purchased from C. Lennig & Co. Ltd., London; Drazine (1-methyl-2-phenoxyethylhydrazine hydrogen maleate) was supplied by Smith & Nephew Research Ltd., Harlow, Essex. A purified preparation of 2-phenylindole was kindly provided by Professor R. Truhaut.

*Animals.* Albino rats, bred from specific-pathogen-free stock, were obtained from the Chemical Defence Experimental Establishment, Porton Down, Wiltshire; other rats used

were Charles River, Carworth and MRC Carshalton stock (originally from Porton). The diets were either MRC diet no. 41 B or Spiller's. The rats were housed 2–6 to a cage.

*Treatment.* Carbon tetrachloride, allyl alcohol and safrole, were injected subcutaneously as single doses in rats, which were killed at appropriate times; phenobarbitone and sodium barbitone were given either subcutaneously in a single dose or by oral intubation daily for several days; all the other compounds were administered by intubation daily for various lengths of time and animals were killed 24 hr after the last treatment. The volume of solution administered to each rat was 0·4–0·5 ml/100g. Aflatoxin was dissolved in dimethylsulphoxide and 0·1 ml of the solution given daily, with 0·1 ml of solvent for control animals.

*Vehicles.* Carbon tetrachloride, allyl alcohol, safrole and dimethylsulphoxide were administered undiluted. All the other compounds were given by intubation as solutions or suspensions in olive oil, with the exception of Triton X-100, which was dissolved in water and phenobarbitone and sodium barbitone which were administered in physiological saline solution.

*Preparation of liver homogenates.* Rats were killed by asphyxiation using nitrogen. The vena cava was opened and blood drained out. The liver was quickly removed, weighed and transferred to a Potter-Elvehjem homogenizer placed in an ice bath. A 25% (w/v) homogenate was prepared by grinding the tissue in the presence of ice-cold 0·25 M-sucrose, 0·001 M-EDTA solution, pH 7·2–7·4, with a Teflon pestle, clearance 0·02 in. running at 1000–1300 rpm.

*Preparation of liver particulate fractions.* The liver cell organelles were separated from the homogenate at 0°C by differential centrifugation using an MSE "Highspeed 17" centrifuge. Expressed in terms of the composite unit g-min (de Duve & Berthet, 1953) the conditions of fractionation were as follows: nuclei $6\times10^3$; mitochondria, $3{\cdot}3\times10^4$; lysosomes, $2{\cdot}5\times10^5$; microsomes, $3\times10^6$.

*Enzyme assays.* Published methods were suitably modified.

*Mitochondria.* Succinic dehydrogenase, determined by the method of Ells (1959) using phenazine methosulphate and dichlorophenolindophenol, proved satisfactory with a saturated solution of phenazine methosulphate as recommended by Arrigoni & Singer (1962) and the enzyme activation procedure of Johnson (1962). The incubation medium was 0·8 mg phenazine methosulphate, 5 mM phosphate buffer pH 7·6, 10 mM KCN, 20 mM sodium succinate, 25 mM dichlorophenolindophenol.

After incubation at 25°C, the reduction of dichlorophenolindophenol was measured by the fall in optical density at 600 mμ.

*Lysosomes.* Acid phosphatase was determined by measuring the amount of inorganic P liberated in the presence of 50 mM sodium glycerophosphate and 100 mM citrate buffer pH 5·8 (Golberg, Martin & Leigh, 1962). Inorganic P was determined according to Berenblum & Chain (1938 a,b).

β-Glucuronidase was assayed by the method of Talalay, Fishman & Huggins (1946) as modified by Kerr & Levvy (1951). The incubation was performed in the presence of 1·25 mM phenolphthaleinglucuronide and 75 mM acetate buffer pH 5·2.

Cathepsin was determined by the method of Anson (1937). The incubation mixture contained 26 mM methaemoglobin and 100 mM acetate buffer pH 3·6.

Arylsulphatase was assayed by the method of Roy (1953) and was slightly modified by carrying out the incubation in a mixture of 75 mM 2-hydroxy-5-nitrophenyl sulphate and 100 mM acetate buffer pH 5·5.

*Microsomes.* Glucose 6-phosphatase was assayed according to Appelmans, Wattiaux & de Duve (1955) in a medium containing 3 mM glucose 6-phosphate, 7 mM histidine buffer pH 6·5 or 80 mM cacodylate buffer pH 6·5 and 1 mM versene.

Azo reductase was measured by using a food colouring, Brown FK. This is stated to be a mixture of the sodium salt of 1,3-diamino-4,6-di(*p*-sulphophenylazo)benzene and the sodium salt of 2,4-diamino-5-(*p*-sulphophenylazo)toluene. It was demonstrated that the effect of rat-liver homogenate on Brown FK was to render it colourless. The apparent reductive cleavage of these compounds was found to be NADP-dependent with an optimum pH of 6·7–7·2. The azo compounds were incubated with homogenate in the presence of 0·2 μM NADP, 0·2 μM NAD, 100 μM nicotinamide, 30 μM glucose 6-phosphate and 200 μM phosphate buffer, pH 6·7, according to Fouts, Kamm & Brodie (1957). At the end of the incubation the reaction was stopped by the addition of 10% NaOH and the decrease in the optical density measured at 462 mμ.

*Cytoplasm.* Glucose 6-phosphate dehydrogenase was assayed by the method of Balinsky & Bernstein (1963) using 2 mM glucose 6-phosphate, 0·3 mM NADP and 100 mM tris buffer, pH 8·2. After incubation at 25°C, the increase in optical density at 340 mμ was measured.

*Protein.* This was determined by the technique of Lowry, Rosebrough, Farr & Randall (1951).

*Enzyme units.* The results of measurements of enzyme activity are expressed in units, representing the decomposition of 1 μmole of substrate/hr/mg protein under the conditions of assay.

## RESULTS

### *Effect of hepatotoxic compounds on lysosomal enzyme activity*

In the first instance, the highly hepatotoxic compounds carbon tetrachloride, allyl alcohol and safrole were administered parenterally. Table 1 shows the effect of these compounds on the activities of some lysosomal enzymes. Of the 4 liver enzymes measured, only 2 were affected by treatment of the rats with the hepatotoxic compounds. Carbon tetrachloride and allyl alcohol caused a substantial increase in the activity of acid phosphatase when measurements were carried out at appropriate times after administration. An increase in the same enzyme was found in individual rats treated with safrole. Arylsulphatase was also raised but β-glucuronidase and cathepsin were practically unchanged, except in rats treated with allyl alcohol, where β-glucuronidase was increased.

The activity of these lysosomal enzymes was also studied in response to different doses of coumarin given by intubation or incorporated in the diet. Coumarin was found to be hepatotoxic at relatively high doses (Hazleton, Tusing, Zeitein, Thiessen & Murer, 1956). The results of these experiments are recorded in Table 2. Here again only the activity of acid phosphatase and arylsulphatase was affected by treatment with a toxic agent. Indeed arylsulphatase was only increased at high dose levels.

It has been reported that intraperitoneal injections of sodium barbitone and phenobarbitone are followed by a rise in the rate of ascorbic acid synthesis, reflecting stimulation of liver microsomal enzyme activities (Burns, Evans & Trousof, 1957). Simultaneously there is an increase in the weight of the liver. Since barbitone is neither metabolized nor accumulated in the body, but is excreted unchanged, it is a particularly suitable test substance

Table 1. *Effect of hepatotoxic agents on the lysosomal enzymes of rat liver*

| Substance | Dose (ml/kg) | Time of killing (hr) | Sex | Acid phosphatase | β-Glucuronidase | Cathepsin | Arylsulphatase |
|---|---|---|---|---|---|---|---|
| Control, olive oil | 0·5–1·5 | 0·5–16 | Male | 0·148±0·010 | 3·34±0·32 | 2·12±0·19 | 6·11±0·58 |
| | | | Female | 0·127±0·014 | 3·87±0·29 | 2·30±0·17 | 6·03±0·61 |
| Carbon tetrachloride | 0·5–1·5 | 10–16 | Male | 0·182±0·030 | 3·19±0·23 | 2·05±0·31 | 6·83±0·84 |
| | | | Female | 0·193±0·013* | 3·69±0·23 | 1·95±0·25 | 8·05±0·71* |
| Allyl alcohol | 0·5–1·5 | 0·5–1·5 | Male | 0·181±0·022* | 4·50±0·28* | 2·18±0·18 | 4·87±0·61 |
| | | | Female | 0·216±0·028** | 4·45±0·37 | 2·03±0·21 | 6·62±0·52 |
| Safrole | 0·5–1·5 | 2·5–3 | Male | 0·202±0·035 | 3·50±0·41 | 2·65±0·29 | 5·88±0·43 |
| | | | Female | 0·241±0·049 | 3·61±0·39 | 2·07±0·20 | 6·25±0·55 |

Rats: Porton SPF and Carshalton strain; body weight: males 150–310 g, females 100–180 g. Treatment: intraperitoneal. Results are given in units as means ±SD. Each value represents 6–16 rats given 0·5, 1·0 or 1·5 ml of the hepatotoxic compound/kg body weight. Other details are given in the test. Values marked with asterisks differ significantly from those of controls: *$P<0·05$; **$P<0·02$.

Table 2. *Activity of lysosomal enzymes in the liver of coumarin-treated rats*

| Enzyme | Sex | Dietary level for 9 days–6 months (%) | | | Oral intubated doses for 1 week (mg/kg/day) | |
|---|---|---|---|---|---|---|
| | | 0 | 0·2 | 1·0 | 0 | 100–300 |
| Acid phosphatase | Male | 0·136±0·009 | 0.179±0·030* | 0·242±0·006** | 0·131±0·007 | 0·169±0·020 |
| | Female | 0·123±0·011 | 0·118±0·020 | 0·203±0·022* | 0·118±0·006 | 0·202±0·013** |
| β-Glucuronidase | Male | 2·60 ±0·18 | 3·33 ±0·41 | 3·39 ±0·32 | 3·12 ±0·27 | 3·44 ±0·10 |
| | Female | 3·27 ±0·22 | 3·32 ±0·11 | 3·71 ±0·18 | 2·82 ±0·18 | 3·20 ±0·13 |
| Cathepsin | Male | 2·15 ±0·17 | 3·17 ±0·42 | 3·42 ±0·55 | 2·15 ±0·22 | 2·55 ±0·27 |
| | Female | 2·51 ±0·27 | 2·47 ±0·32 | 3·10 ±0·15 | 2·27 ±0·22 | 2·65 ±0·18 |
| Arylsulphatase | Man | 6·15 ±0·58 | 8·01 ±1·49 | 14·01 ±1·84** | 5·84 ±0·63 | 7·01 ±1·06 |
| | Female | 6·51 ±0·52 | 8·50 ±0·55 | 12·82 ±1·57** | 5·95 ±0·14 | 7·08 ±0·91* |

Rats: Porton SPF strain, initial weight 60–100 g. Results are given in units as means ±SD. Each value represents 6–12 rats. Other details are given in the text. Values marked with asterisks differ significantly from those of controls: *$P<0·05$; **$P<0·02$.

for assessing the response of lysosomal enzymes to a compound which is not known to be hepatotoxic. It is seen from Table 3 that daily injections of sodium barbitone did not result in any significant change of the lysosomal enzymes assayed.

Table 3. *Activity of lysosomal enzymes in the liver of control and sodium barbitone-treated rats*

| Enzyme | Duration of treatment (days) | | | |
|---|---|---|---|---|
| | 9 | | 16 | |
| | Control | Barbitone | Control | Barbitone |
| Acid phosphatase | 0·116±0·010 | 0·129±0·011 | 0·114±0·008 | 0·145±0·007 |
| β-Glucuronidase | 2·76 ±0·19 | 2·40 ±0·36 | 2·98 ±0·28 | 2·24 ±0·46 |
| Cathepsin | 2·05 ±0·18 | 2·12 ±0·20 | 2·95 ±0·32 | 2·23 ±0·27 |
| Arylsulphatase | 5·89 ±0·57 | 5·45 ±0·58 | 7·24 ±0·52 | 6·06 ±0·55 |

Rats: Porton SPF female weighing 200–250 g. Treatment: intraperitoneal, 200 mg sodium barbitone/kg body weight in 5 ml physiological saline solution daily. Results are given in units as means±SD and represent 6–10 rats. Other details are given in the text.

*Effect of hepatotoxic compounds on microsomal enzyme activity*

Glucose 6-phosphatase and azo reductase have been assayed as representative liver microsomal enzymes. Azo reductase is also present in the lysosomal fraction of the rat-liver cell (Fouts *et al.* 1957).

The glucose 6-phosphatase activity of liver homogenate was greatly reduced following carbon tetrachloride injections and in most of the rats given allyl alcohol and safrole. Table 4 presents these results. The reduction in activity of glucose 6-phosphatase brought about by carbon tetrachloride has been previously reported by Recknagel & Lombardi (1961) and by Börnig, Horn & Mücke (1962). Although azo reductase was also decreased in the liver of rats treated with allyl alcohol, this did not occur in coumarin-treated rats. On the other hand glucose 6-phosphatase activity was greatly reduced by high levels of coumarin.

Table 4. *Activity of microsomal enzymes in the liver of rats treated with several hepatotoxic compounds*

| Compound administered | Dose | Route | Time of killing | Enzyme | |
|---|---|---|---|---|---|
| | | | | Glucose 6-phosphatase | Azo-reductase |
| None | | | | 1·91±0·30 | |
| Physiological saline | 0·5–1·5 ml/kg | Intraperitoneal | 0·5–16 hr | 1·78±0·21 | 1·84±0·26 |
| Olive oil | | Oral | 8 days | 1·76±0·19 | |
| Carbon tetrachloride | 0·5–1·5 ml/kg | Intraperitoneal | 10–16 hr | 0·78±0·24*** | 1·73±0·21 |
| Allyl alcohol | 0·5–1·5 ml/kg | Intraperitoneal | 0·5– 1 hr | 1·18±0·39** | 0·87±0·18** |
| Safrole | 0·5–1·5 ml/kg | Intraperitoneal | 2·5–3 hr | 1·39±0·27* | 2·11±0·22 |
| Coumarin | 200 mg/kg/day | Oral | 8 days | 1·25±0·33** | 1·61±0·13 |
| Coumarin | 0·2% | Diet | 9 days–6 months | 1·34±0·42 | |
| Coumarin | 1·0% | Diet | do. | 0·91±0·26*** | |

Rats: Porton SPF and Carshalton females, initial weight: 80–100 g. Results are given in units as means± SD. Azo-reductase: 1 unit=1 μg Brown FK reduced/hr/mg protein at 37 C. Each value represents 6–15 rats. Other details are given in the text. Values marked with asterisks differ significantly from those of controls: *$P<0·05$; **$P<0·02$; ***$P<0·01$.

*Effect of hepatotoxic compounds on mitochondrial and cytoplasmic enzymes*

The activity of the mitochondrial enzyme, succinic dehydrogenase, was unaffected by the different compounds administered to rats. However, some of these substances elicited a substantial rise in the activity of glucose 6-phosphate dehydrogenase in the cytoplasmic fraction (Table 5).

*Table 5. Activity of succinic and glucose 6-phosphate dehydrogenases in the liver of rats treated with various compounds*

| Compound administered | Dose (ml/kg) | Route | Time of killing | Enzyme: Succinic dehydrogenase | Enzyme: Glucose 6-phosphate dehydrogenase |
|---|---|---|---|---|---|
| None | | | | 0·189±0·022 | 0·195±0·024 |
| Physiological saline | 0·5–1·5 | Intraperitoneal | 0·5–1 hr | | 0·276±0·021 |
| Carbon tetrachloride | 0·5–1·5 | Intraperitoneal | 10–16 hr | 0·127±0·019 | 0·546±0·036** |
| Allyl alcohol | 0·5–1·5 | Intraperitoneal | 0·5–1 hr | 0·172±0·023 | 0·183±0·042 |
| Safrole | 0·5–1·5 | Intraperitoneal | 2·5–3 hr | 0·112±0·021 | 0·318±0·033 |
| Sodium barbitone | 200 mg/kg | Intraperitoneal | 9 days | 0·198±0·014 | 0·261±0·030 |
| | | | 16 days | 0·165±0·020 | 0·219±0·018 |
| Coumarin | 1% | Diet | 9 days | 0·180±0·012 | 0·363±0·033* |
| | | | 16 days | 0·244±0·011 | 0·585±0·027*** |

Rats: Porton SPF strain, initial weight 80–100 g. Results are given in units as means±SD. Each value represents 4–8 rats. Other details are given in the text. Values marked with asterisks differ significantly from those of controls: **$P<0·02$; ***$P<0·01$.

*Effect of various compounds on glucose 6-phosphatase and glucose 6-phosphate dehydrogenase*

It appears from the investigations described above that glucose 6-phosphatase and glucose 6-phosphate dehydrogenase in particular were affected by hepatotoxic agents. Accordingly, a wider study was carried out with various hepatotoxic substances, with hepatocarcinogens and a variety of polar and non-polar compounds, some of them not known to produce liver damage. The levels of enzyme activity and changes in relative liver weight are recorded in Table 6.

It is seen from Table 6 that the activity of liver glucose 6-phosphatase was greatly reduced following treatment with hepatotoxic compounds, but the majority of the compounds not known to affect the liver brought about no change in enzyme level. Glucose 6-phosphate dehydrogenase activity and liver weight were increased in some of the animals treated with toxic compounds but did not undergo change when non-hepatotoxic compounds were administered.

Arising from these exploratory studies, it was considered to be of interest to investigate the correlation between an increase in relative liver weight and the effect on enzyme activity brought about by two of the compounds studied. Butylated hydroxytoluene and 2-phenylindole were chosen as substances capable of inducing liver enlargement at high doses, while dimethylsulphoxide was used as a control material. The results in Table 7 provide evidence that very high doses of dimethylsulphoxide had no effect on either glucose 6-phosphate metabolism or on liver weight. With butylated hydroxytoluene, a significant increase in relative liver weight was observed after 7 daily doses of 100 mg/kg in males and 200 mg/kg in females. Yet it was only with doses of 500 mg/kg that striking changes occurred in the

Table 6. *Glucose 6-phosphatase, glucose 6-phosphate dehydrogenase and liver weight of rats treated with various compounds*

| Compound administered | Dose/kg body weight | Route | Time of killing | Glucose 6-phosphatase | Glucose 6-phosphate dehydrogenase | Relative liver weight (%) |
|---|---|---|---|---|---|---|
| | | | CONTROL | | | |
| None | | | | 1·71±0·16 | 0·402±0·033 | 4·68±0·39 |
| | | | HEPATOTOXIC COMPOUNDS | | | |
| Carbon tetrachloride | 0·5-1·5 ml | Intraperitoneal | 6-16 hr | 1·00±0·11*** | 1·134±0·057*** | |
| Safrole | 0·5-1·5 ml | Intraperitoneal | 2·5-3 hr | 1·54±0·11* | 0·369±0·270 | |
| Allyl alcohol | 0·5-1·5 ml | Intraperitoneal | 0·5-1 hr | 1·35±0·13* | 0·438±0·039 | |
| Coumarin | 200 mg | Oral | 7 days | 1·17±0·09** | 0·729±0·048*** | 5·88±0·34** |
| β-Thujone | 100 mg | Oral | 7 days | 1·04±0·08*** | 0·336±0·036 | 5·15±0·40 |
| Ethionine | 200 mg | Oral | 7 days | 1·08±0·13** | 0·726±0·051** | 5·26±0·43 |
| Thioacetamide | 60 mg | Oral | 3-7 days | 1·19±0·12** | 0·438±0·057 | 5·45±0.49 |
| 3-Methyl-4'-dimethylamino-azobenzene | 150 mg | Oral | 7 days | 0·95±0·09*** | 0·447±0·030 | 4·97±0·37 |
| Dimethylnitrosamine | 2 mg | Oral | 7 days | 1·54±0·11* | 0·825±0·033*** | 5·13±0.35 |
| | 10 mg | Oral | 7 days | 1·13±0·12** | 0·825±0·054** | 4.72±0.40 |
| Aflatoxin | 0·5 mg | Oral | 6 days | 1·32±0·10* | 0·234±0·027 | 4·23±0·33 |
| | | | NON-HEPATOTOXIC COMPOUNDS | | | |
| Dimethylsulphoxide | 1·0 ml | Oral | 6 days | 1·62±0·18 | 0·294±0·024 | 4·55±0·41 |
| Triton X-100 | 250 mg | Oral | 3-7 days | 1·82±0·19 | 0·285±0·030 | 5·57±0·24 |
| Stanclere 176 (Dioctyltin) | 250 mg | Oral | 3-7 days | 2·19±0·20 | 0·204±0·024 | 5·41±0·54 |
| Diazine [(1-methyl-2-phenoxyethyl) hydrazine hydrogen maleate] | 125 mg | Oral | 3-7 days | 1·69±0·21 | 0·282±0·009 | 5·45±0·51 |
| Glyceroglyceride | 200 mg | Oral | 7 days | 2·14±0·19 | 0·228±0·030 | 5·08±0·26 |
| Butylated hydroxytoluene | 500 mg | Oral | 4 days | 1·49±0·46 | 0·486±0·033 | 6·15±0·31*** |
| Butylated hydroxyanisole | 500 mg | Oral | 4 days | 2·02±0·17 | 0·435±0·036 | 4·61±0·38 |
| 2-Phenylindole | 250 mg | Oral | 7-14 days | 1·14±0·15*** | 0·459±0·015 | 6·26±0·22*** |
| Sodium barbitone | 200 mg | Intraperitoneal | 9-16 days | 2·15±0·14 | 0·243±0·030 | 5·50±0·24 |

Wistar Porton SPF and Carshalton females, initial weight 60–150 g. Results are given in units as means ±SD. Each value represents 6–22 rats. Other details are given in the text. Values marked with asterisks differ significantly from those of controls: *$P<0·05$; **$P<0·02$; ***$P<0·01$.

Table 7. *Comparison of changes in liver enzyme activities and relative liver weight brought about by various doses of three compounds.*

| Compound tested | Doses given daily for 7 days (mg/kg) | Males | | | Females | | |
|---|---|---|---|---|---|---|---|
| | | Glucose 6-phosphatase | Glucose 6-phosphate dehydrogenase | Relative liver weight (%) | Glucose 6-phosphatase | Glucose 6-phosphate dehydrogenase | Relative liver weight (%) |
| Dimethylsulphoxide | 0 | 1·88±0·19 | 0·495±0·126 | 5·29±0·40 | 1·64±0·15 | 0·423±0·054 | 5·27±0·48 |
| | 1100 | 1·66±0·18 | 0·315±0·093 | 5·36±0·31 | 1·62±0·18 | 0·296±0·054 | 4·55±0·41 |
| | 3300 | 1·73±0·15 | 0·453±0·051 | 5·91±0·34 | 1·97±0·27 | 0·315±0·117 | 5·28±0·39 |
| | 11,000 | 1·96±0·21 | 0·435±0·069 | 5·16±0·76 | 1·67±0·24 | 0·354±0·101 | 5·25±0·33 |
| Butylated hydroxytoluene | 0 | 1·67±0·16 | 0·561±0·075 | 4·26±0·34 | 1·50±0·20 | 0·430±0·125 | 4·74±0·29 |
| | 50 | 1·47±0·20 | 0·552±0·114 | 4·88±0·40 | 1·36±0·27 | 0·477±0·177 | 4·82±0·21 |
| | 100 | 1·56±0·41 | 0·489±0·097 | 5·74±0·25*** | 1·42±0·12 | 0·489±0·096 | 5·54±0·37 |
| | 200 | 1·59±0·12 | 0·594±0·156 | 5·84±0·25*** | 1·34±0·26 | 0·585±0·120 | 6·16±0·44** |
| | 500 | 1·19±0·27** | 1·224±0·315*** | 6·77±0·21*** | 0·83±0·27*** | 1·233±0·222*** | 6·17±0·51*** |
| 2-Phenylindole | 0 | 1·95±0·25 | 0·339±0·051 | 5·16±0·49 | 1·44±0·10 | 0·387±0·057 | 4·99±0·37 |
| | 10 | 1·83±0·20 | 0·288±0·084 | 6·08±0·71* | 1·26±0·18 | 0·573±0·099 | 5·41±0·54 |
| | 25 | 1·69±0·21 | 0·531±0·102 | 5·72±0·47 | 1·08±0·13* | 0·501±0·120 | 5·54±0·24 |
| | 100 | 1·45±0·11** | 0·306±0·087 | 6·07±0·20** | 1·01±0·13** | 0·462±0·042 | 6·31±0·22*** |
| | 250 | 1·23±0·18*** | 0·414±0·036 | 6·24±0·19*** | 0·97±0·15** | 0·459±0·045 | 6·28±0·22*** |

Rats: Carworth, initial weight 80–120 g. Treatment: oral doses of undiluted dimethylsulphoxide, butylated hydroxytoluene and 2-phenylindole in 5 ml olive oil/kg. Results are given in units as means ±SD. Each value represents 6 rats. Values marked with asterisks differ significantly from those of controls: *$P<0·05$; **$P<0·02$; ***$P<0·01$.

levels of glucose 6-phosphatase and glucose 6-phosphate dehydrogenase. Similarly, in the case of 2-phenylindole, an increase in relative liver weight was seen in males on doses of 10 mg/kg; 25 mg/kg caused an increase that did not reach the level of significance but with 100 and 250 mg/kg the relative liver weight was significantly raised above normal in females as well as males. Glucose 6-phosphatase showed a distinct fall in females at 25 mg/kg and in males at 100 mg/kg. An effect on glucose 6-phosphate dehydrogenase was not observed.

## DISCUSSION

*Lysosomal enzymes*

Investigations by de Duve, Pressman, Gianetto, Wattiaux & Appelmans (1955) and de Duve (1959) provided the first evidence linking lysosomal hydrolases with various processes of autolysis and necrosis. It has been suggested that perforation or rupture of the lysosomal membrane is followed by simultaneous release of bound enzymes in soluble and fully active form. Consistent with this idea, increased activity of acid phosphatase and other lysosomal enzymes were found by Golberg, Martin & Batchelor (1960) when iron dextran complex was injected in high doses into mice and rats. There are indications that rupture of lysosomes may be a possible source of cell damage in vitamin A intoxication (Lucy, Dingle & Fell, 1961), after exposure to streptolysin (Hirsch, Bernheimer & Weissmann, 1963), and possibly in some other cases. The lysosomal enzymes presumably manufactured at the ribosomes can gain access to the endoplasmic reticulum, and thence be transferred to the Golgi apparatus, and further to secondary lysosomes such as multivesicular bodies and dense bodies (Novikoff, Essner & Quintana, 1964). The biochemical assay of lysosomal enzymes cannot distinguish between primary and secondary lysosomes and between true intracellular rupture of lysosomes and transfer of the lysosomal enzymes to larger or more fragile vacuoles such as those created when phagocytosis is stimulated (Hirsch & Cohn, 1964).

It has been observed that damage to the lysosomes do not always result in parallel effects on the lysosomal enzymes. An increased activity of all the lysosomal enzymes studied was found in muscle following muscular dystrophy due to vitamin E deficiency (Tappel, Zalkin, Caldwell, Desai & Shibko, 1962; Zalkin, Tappel, Caldwell, Shibko, Desai & Holliday, 1962). However, the response to liver enzymes was different: only cathepsin and arylsulphatase were altered by this treatment. The possibility arises that changes in lysosomal enzymes may be secondary to primary effects on the rough endoplasmic reticulum, on the Golgi apparatus or on autophagic vacuoles. Our own experience supports this suggestion. It has been found that toxic liver injury did not bring about parallel changes in the activities of the 4 lysosomal enzymes tested. Acid phosphatase was raised following the administration of carbon tetrachloride, coumarin or allyl alcohol and in individual animals given safrole. Increased activity of this enzyme appeared only at doses that were higher than those inducing changes in glucose 6-phosphatase or liver enlargement. Arylsulphatase was increased only in rats treated with carbon tetrachloride and coumarin. $\beta$-Glucuronidase and cathepsin were unchanged during intoxication with carbon tetrachloride, coumarin or safrole, but when allyl alcohol was injected $\beta$-glucuronidase was raised. Sodium barbitone did not elicit any effect on the lysosomal enzymes tested.

*Mitochondrial and cytoplasmic enzymes*

Succinic dehydrogenase activity was similar in the liver of control and treated animals and did not provide any indication of toxic effect of these compounds. The activity of the

plasmic enzyme, glucose 6-phosphate dehydrogenase, was altered by some hepatotoxic agents. Carbon tetrachloride, coumarin, dimethylnitrosamine and ethionine greatly increased the activity of this enzyme and a rise in the liver glucose 6-phosphate dehydrogenase was found in more than 50% of the rats given allyl alcohol and safrole; but no change in activity observed when β-thujone, 3-methyl-4′-dimethylazobenzene, thioacetamide or aflatoxin were administered. This change seen in the activity of glucose 6-phosphate dehydrogenase may be a consequence of disturbed glucose 6-phosphate metabolism.

*Microsomal enzymes*

Fouts, Dixon & Shultice (1961) and Dixon, Hart & Fouts (1961) demonstrated that several processing enzymes which metabolize foreign compounds, were almost exclusively localized in the smooth-surfaced microsomes derived from the smooth-surfaced endoplasmic reticulum. Following the administration of carbon tetrachloride to rats, Reynolds (1963) found that toxic damage to the liver was accompanied by dispersal of the ergastoplasm, and noted a striking reduction in the activity of glucose 6-phosphatase demonstrable histochemically in the livers of these animals. In our experiments a consistent pattern was found in the effect of hepatotoxic compounds on the activity of glucose 6-phosphatase assayed biochemically. All the hepatotoxic compounds administered (carbon tetrachloride, safrole, allyl alcohol, coumarin, β-thujone, ethionine, thioacetamide, 3-methyl-4′-dimethylaminoazobenzene, dimethylnitrosamine, aflatoxin) brought about a highly significant fall in the activity of this enzyme. Various other compounds, none of them known to be toxic to the liver, brought about no change in liver glucose 6-phosphatase. Among these substances was sodium barbitone, a well-known stimulator of microsomal "processing" enzymes.

Tests were carried out on some compounds of questionable hepatotoxicity. Dimethylsulphoxide given to rats did not elicit any fall in glucose 6-phosphatase activity. This compound did not show any toxicity (Brown, Robinson & Stevenson, 1963). On the other hand, 2-phenylindole which is not known to be hepatotoxic, proved capable of bringing about a reduction in enzyme activity at high doses. Daily doses of 100–500 mg butylated hydroxytoluene given to rats for 7 days induced liver enlargement but glucose 6-phosphatase was reduced only at the 500 mg/kg level.

Two questions arise: is the reduced activity of glucose 6-phosphatase related to structural changes in the endoplasmic reticulum, and is the falling-off of enzyme activity indicative of hepatic damage? It is known that changes in liver glucose 6-phosphatase activity occur in various physiological and pathological circumstances. Stress elicits an increase in enzyme activity. Synthesis of the enzyme is induced by administration of cortisone (Weber & Singhal, 1964); a slight rise in glucose 6-phosphatase activity is brought about by fasting; activity of the enzyme is greatly increased in the diabetic liver (Ashmore, Hastings & Nesbett, 1954; Ashmore, Cahil, Hastings & Zottu, 1957). On the other hand, lowering of the enzyme level in the liver of rats takes place during the development of various tumours (Ashmore, Weber & Landau, 1958; Albert & Orlowski, 1960; Pitot 1960), in the liver of rats fed with butter yellow (Weber & Cantero, 1955), nitrosomorpholine (Friedrich-Freksa, Süss, Lanka & Börner, 1964) or diethylnitrosamine (Heise & Görlich, 1964). Our results are in agreement with those of Heise & Görlich (1964). Taken in conjunction with morphological studies, our results suggest a relationship between the structure of the reticulum and activity of glucose 6-phosphatase.

An increase in the relative liver weight was brought about by most of the hepatotoxic

compounds studied. This phenomenon is generally considered to reflect the toxic effects of such compounds. However, taking our results as a whole, it is clear that if a fall in glucose 6-phosphatase activity is indicative of liver damage, it constitutes a more sensitive index of toxicity than an increase in relative liver weight.

Glucose 6-phosphatase plays a very important role in carbohydrate metabolism. The decreased enzyme activity which was found after treatment with hepatotoxic compounds as measured under optimal conditions seems to indicate the presence of a non-functioning or decreased enzyme population of the tissue. This may produce various changes in the amount or activity of other liver enzymes and subsequently pathological conditions arise. It is difficult to conceive any structural relationship between the various compounds used apart from lipid solubility of most of them. Therefore, it is possible that the fall in glucose 6-phosphatase is also a result of subsequent changes following variations in the activity of other enzymes altered primarily by poisoning of the liver.

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## Tests de *Réactivité Hépatique*. I. Recherches sur la *Glucose-6-phosphatase* et sur d'Autres Enzymes Hépatiques

***Résumé***—*Le but des expériences fut de détecter des modifications dans l'activité de quelques systèmes enzymatiques de la cellule hépatique du rat sous l'effet de différents composés chimiques.*

On étudia les enzymes hépatiques suivantes:
—mitochondries: déshydrogénase succinique:
—microsomes: glucose-6-phosphatase, azo réductase;
—lysosomes: phosphatase acide, β-glycuronidase, cathepsine, arylsulphatase;
—cytoplasme: déshydrogénase du glucose-6-phosphate.

On compara des rats témoins à des rats auxquels on avait fait absorber des composés hépatotoxiques (tétrachlorure de carbone, safrole, alcool allylique, coumarine, β-thuyone, éthionine, sulfacétamide, méthyl-3-diméthylamino-4-azo-benzène, diméthylnitrosamine, aflatoxine); des additifs alimentaires (butylate d'hydroxyanisole, butylate d'hydroxytoluène, sucroglycéride); des stabilisateurs (dioctyltine, phényle-2-indole); des médicaments (Drazine, barbital sodique) et d'autres composés (Triton X-100, diméthylsulfoxide).

Une diminution caractéristique de l'activité de la glucose-6-phosphatase s'observa après administration d'une quantité convenable de l'un quelconque des composés hépatotoxiques pendant un temps approprié. De même, le phényl-2-indole et le butylate d'hydroxytoluène donnés à haute dose amenèrent un affaiblissement de l'activité de cette enzyme. Les autres composés ne provoquerent pas de diminution significative de la glucose-6-phosphatase.

L'activité de la déshydrogénase du glucose-6-phosphate s'accrut après l'administration à des rats de tétrachlorure de carbone, de coumarine, d'éthionine, de diméthylnitrosamine, de phényl-2-indole et de hautes doses de butylate d'hydroxytoluène.

L'activité des différentes enzymes lysosomiques ne varia pas uniformément. Après la prise de composés hépatotoxiques, le taux de la phosphatase acide et de l'arylsulphatase s'éleva, tandis qu'il n'y eut aucun changement marqué en ce qui concerne la β-glycuronidase et la cathepsine. L'absorption de barbital sodique n'entraina aucune variation des enzymes des lysosomes.

La déshydrogénase succinique des mitochondries hépatiques de rats traités par différents composés chimiques ne subit aucune modification d'activité.

## Prüfungen der Leberreaktion. I. Voruntersuchungen an Glucose-6-phosphatase und Anderen Leberenzymen

**Zusammenfassung**—Die Untersuchungen hatten die Aufgabe Aktivitätsänderungen einiger Enzymsysteme in den Leberzellen von Ratten nach Verabreichung verschiedener Verbindungen festustellen.

Die folgenden Leberenzymsysteme wurden untersucht: Mitochondrien: Succinatdehydrogenase; Mikrosomen: Glucose-6-phosphatase, Azoreduktase; Lysosomen: Saure Phosphatase, β-Glucuronidase, Kathepsin, Arylsulfatase; Cytoplasma: Glucose-6-phosphatdehydrogenase.

Unbehandelte Ratten wurden mit solchen verglichen, die mit hepatotoxischen Verbindungen (Tetrachlorkohlenstoff, Safrol, Allylalkohol, Cumarin, β-Thujon, Äthionin, Thioacetamid, 3-Methyl-4-dimethylamineazobenzol, Dimethylnitrosamin, Aflatoxin), mit Nahrungsmittelzusätzen(Butyliertes Hydroxyanisol, Butyliertes Hydroxytoluol, Sucroglycerid), mit Stabilisatoren (Dioctylzinn, 2-Phenylindol), mit Medikamenten (Drazin, Natriumdiäthylbarbiturat)und anderen Verbindungen(Triton X-100, Dimethylsulfoxyd) behandelt worden waren.

Eine signifikante Verminderung der Glucose-6-phosphatase-Aktivität wurde beobachtet, wenn eine der hepatotoxischen Verbindungen in entsprechender Dosierung über längere Zeit verabreicht wurde. Ähnlich verursachten auch 2-Phenylindol und butyliertes Hydroxytoluol in hoher Dosierung eine Verminderung der Aktivität dieses Enzyms. Die anderen Verbindungen verursachten keine signifikante Verminderung der Glucose-6-phosphatase-Aktivität.

Die Glucose-6-phosphatdehydrogenase-Aktivität war erhöht nach der Behandlung der Ratten mit Tetrachlorkohlenstoff, Cumarin, Äthionin, Dimethylnitrosamin, 2-Phenylindol und hohen Dosen von butyliertem Hydroxytoluol.

Die Aktivitätsänderungen der untersuchten lysosomalen Enzyme gingen nicht parallel. Nach der Anwendung der hepatotoxischen Verbindungen vermehrten sich saure Phosphatase und Arylphosphatase, während β-Glucuronidase und Kathepsin keine deutliche Änderung zeigten. Die Behandlung mit Natriumbarbiturat verursachte keine Änderung bei den lysosomalen Enzymen.

Hinsichtlich der Aktivität der Succinatdehydrogenase in den Lebermitochondrien von Ratten, die mit verschiedenen Verbindungen behandelt wurden, war kein Unterschied feststellbar.

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# The Residue of Butylated Hydroxytoluene (BHT) and Metabolites in Tissue and Eggs of Chickens Fed Diets Containing Radioactive BHT

J. P. FRAWLEY

*Hercules Powder Company, Wilmington, Delaware, USA*

and

J. H. KAY and J. C. CALANDRA

*Industrial Bio-Test Laboratories, Inc., Northbrook, Illinois, USA*

(*Received* 22 *June* 1965)

**Abstract**—Radioactive butylated hydroxytoluene (BHT; 3,5-di-*tert*-butyl-4-hydroxytoluene), ring-labelled with $^{14}C$, has been fed to one-day-old chickens for 10 wk at a level of 200 ppm in the total diet. Chickens were sacrificed at 1, 2, 4 and 10 wk and all tissues and organs analyzed for BHT and metabolites. Maximum residue levels were found after 1 wk. At broiler age edible portions contained between 1 and 3 ppm BHT and metabolites. Slightly higher concentrations were found in isolated fat, skin, and digestive organs. Similar diets fed to laying hens for 4 wk resulted in a gradual increase in residue in eggs to 2 ppm after 7 days. This level remained constant for the remainder of the experiment. At sacrifice, the residue in the fat of laying hens was slightly higher than in the broilers, but edible tissues were not significantly different. When considered in the light of all the toxicological data on BHT, these results confirm the safety of the use of BHT up to 200 ppm in chicken feed.

## INTRODUCTION

One of the important uses of butylated hydroxytoluene (BHT; 3,5-di-*tert*-butyl-4-hydroxytoluene), which is beneficial to the consumer, is its use in animal feeds. Added to feeds, it protects the vitamin constituents, nutrients, and plant pigments against oxidative degradation. In addition, the presence of BHT prevents rancidity of the fat content and thereby maintains palatability. In the case of fish meal, an important ingredient of chicken feed, BHT also prevents oxidative heating and spontaneous combustion. The amount of BHT used in these applications is a maximum of 200 ppm in oil vitamin concentrates and 1000 ppm in fish meal. Since these two feed constituents represent only a small portion of the total animal feed, the BHT content of the total diet is always less than 200 ppm.

A few experiments have been conducted to determine the storage and possible accumulation of BHT in the tissues of animals which have been fed diets containing BHT. Francois & Pihet (1960) fed diets containing 1000 ppm BHT to pigs for 4 months and to chickens for 8 wk. At the end of the feeding period samples of fat, muscle, liver and kidney were analyzed chemically for BHT. No BHT was detected in any of the samples except the fat of chickens which contained 60 ppm BHT. Although these authors do not report the sensitivity of the chemical method used, the method employed, as originally described by Anglin, Mahon & Chapman (1956), has a minimum detection level of about 25 ppm.

Daniel & Gage (1965) fed a diet containing 500 ppm BHT to rats for 35 days and analyzed the fat and liver. Using a more sensitive chemical method, approximately 30 ppm of BHT was detected in the fat of male rats and 45 ppm for female rats. The livers contained approximately 3 ppm for males and 1 ppm for females. There was no significant accumulation of

BHT in the fat and liver beyond the first few days of feeding. The method employed relatively specific for BHT and would not detect some metabolites of BHT.

In an effort to determine more precisely the total retention of BHT and metabolites of BHT in the fat and edible tissues of chickens and the excretion of BHT in eggs, additional experiments have been conducted using $^{14}C$-ring-labelled BHT.

## EXPERIMENTAL

*Preparation of radioactive BHT.* A sample of ring-labelled *p*-cresol was obtained from Nuclear Research Chemicals, Inc., Orlando, Florida and converted into BHT by sulphuric acid-catalyzed alkylation of the $^{14}C$ ring with isobutylene in benzene solution. The product was purified by recrystallization from 90:10 (w/w) mixture of isopropanol and water. The radioactive BHT had a specific activity of 0·080 mC/g and was shown to be >99·9% pure by vapour phase chromatography and ultraviolet spectroscopy.

*Experimental design.* In the poultry industry, there are two major types of chicken raising techniques. The first is for the production of chickens (frying or broiling) and the second is for the production of eggs. Consequently the experiment was conducted in two parts to investigate the BHT residue in the edible tissues of young frying and broiling chickens (experiment A) as well as in the eggs and edible tissue of laying hens (experiment B).

*Experiment A.* In this experiment, 8 one-day-old chickens, 4 males and 4 females, were fed commercial "starter feed" for 4 wk and then commercial "broiler feed" for an additional 6 wk, both of which contained 200 ppm of added radioactive BHT. The total fat content of all diets was adjusted to 10% with the addition of lard to facilitate maximum intestinal absorption of the BHT. One chicken of each sex was sacrificed after 1 wk on the diet and a similar number after 2, 4 and 10 wk. At the time of sacrifice, samples of fat, liver, kidney, leg muscle, breast muscle, wing muscle, skin, heart, gizzard, and viscera were collected and examined for radioactivity.

*Experiment B.* In this experiment 4 young adult laying hens were fed a "laying feed" containing 200 ppm of added radioactive BHT for 4 wk. The total fat content of this diet was also adjusted to 10% with lard to facilitate maximum absorption of the BHT. Eggs were collected daily from these hens, and examinations for radioactivity of the eggs made on the following days: 1, 3, 7, 14, 21 and 28. After 4 wk of feeding, the hens were sacrificed and the same tissues as noted in experiment A were examined for radioactivity.

*Radioactivity measurements.* All of the radioassays were performed, after suitable sample treatment, by liquid scintillation counting. All samples were counted with appropriate blanks. All samples were prepared by the Schöniger combustion method and the evolved $CO_2$ was absorbed in a standard liquid scintillator solution. To determine the percentage recovery, radioactive BHT was added to control samples of the various organs and tissues which were subjected to combustion, absorption and radioassay. The recovery ranged from 95·0 to 104·5% for the different tissues.

## RESULTS

Table 1 summarizes the total BHT and BHT metabolites, as found by radioactive counting, present in the various tissues of the young chickens sacrificed at 1, 2, 4 and 10 wk and the older laying chickens sacrificed after 4-wk feeding of 200 ppm BHT.

Table 1. *Average tissue levels of BHT and BHT metabolites after feeding diets containing 200 ppm radioactive BHT to chickens for up to 10 wk*

| | Tissue levels (ppm) in | | | | |
|---|---|---|---|---|---|
| | Young chickens at wk | | | | Laying hens at wk |
| Tissue | 1 | 2 | 4 | 10 | 4 |
| Fat | 11·2 | 7·4 | 2·2 | 3·9 | 17·5 |
| Breast | 2·4 | 1·0 | 0·9 | 0·9 | 0·2 |
| Leg | 2·8 | 1·3 | 1·2 | 0·6 | 1·4 |
| Wing | 2·7 | 2·0 | 1·3 | 0·8 | 1·8 |
| Liver | 5·6 | 4·9 | 5·4 | 4·4 | 6·8 |
| Kidney | 4·4 | 4·0 | 2·8 | 3·6 | 3·9 |
| Heart | 4·6 | 4·0 | 3·0 | 1·3 | 1·0 |
| Skin | 14·1 | 9·1 | 6·1 | 3·5 | 14·3 |
| Gizzard | 9·1 | 7·1 | 6·9 | 0·9 | 4·8 |
| Viscera | 12·1 | 9·1 | 7·0 | 5·7 | 7·1 |

It is obvious from this Table that the major storage of BHT in the body is in the fat and in tissue which contains a high percentage of fat such as skin. It is also apparent that there is no continued build-up of BHT in the tissues after the first few weeks. In fact, the amount of BHT storage is significantly less after 10 wk than after 1 or 2 wk. No significant difference in storage levels was found between male and female chickens.

Table 2 summarizes the total BHT and BHT metabolites present in the eggs layed by hens fed diets containing 200 ppm BHT. Again, it is apparent that after 1 wk, no additional build-up in the BHT level occurs and it remains at approximately 2 ppm.

Table 2. *Average levels of BHT and BHT metabolites in eggs from chickens fed diets containing 200 ppm radioactive BHT for up to 4 wk*

| Days after feeding started | 1 | 3 | 7 | 14 | 21 | 28 |
|---|---|---|---|---|---|---|
| Concentration in eggs (ppm) | 0·2 | 1·0 | 2·5 | 2·1 | 2·3 | 2·2 |

## DISCUSSION

These experiments demonstrate that the edible portions of chickens which have been raised on feed which contains 200 ppm BHT will contain between 1 and 3 ppm of BHT and metabolites. Slightly higher concentrations will be found in isolated fat, skin, digestive organs and viscera. Eggs produced by laying hens fed similar diets will contain approximately 2 ppm BHT and metabolites.

These results are in close agreement with the results of Francois & Pihet (1960) and Daniel & Gage (1965) who fed non-radioactive BHT to pigs and chickens, and rats, respectively, at higher dietary levels. Since each of the chickens ingested approximately 500 mg/kg of radioactive BHT during the 10-wk feeding period, it is obvious that very little BHT and metabolites were retained in the body—less than 1%.

Even if the total concentration in edible tissue and eggs is assumed to be unchanged BHT, these levels are insignificant in view of the extensive old and new toxicological data on BHT which has demonstrated a "no-effect" and safe level for experimental animals of 1000 ppm (Joint FAO/WHO Expert Committee on Food Additives, 1962; Frawley, Kohn, Kay & Calandra, 1965).

These results confirm that the use of BHT in animal feeds, up to a level of 200 ppm in the total feed, is safe beyond any reasonable doubt.

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## Résidu du butylate d'hydroxytoluène (BHT) et de ses métabolites dans les tissus et les oeufs de poules nourries avec des aliments contenant de BHT radio-actif

**Résumé**—Du butylate d'hydroxytoluène radio-actif (BHT; 3,5-di-*tert*-butyl-4-hydroxytoluène), marqué dans le cycle avec du $C_{14}$ a été donné à des poussins depuis l'âge de 1 jour pendant 10 semaines à raison de 200 ppm réparties sur l'ensemble des ailments. Les poussins furent sacrifiés à 1, 2, 4 et 10 semaines et on dosa dans tous les tissus le BHT et ses métabolites. On trouva les taux les plus élevés de résidu à la fin de la première semaine. Chez la poule adulte, les parties consommables contenaient entre 1 et 3 ppm de BHT et de ses métabolites. On en trouva des proportions un peu plus élevées dans certaines parties comme la graisse, la peau et les organes digestifs. Une alimentation semblable donnée à des poules pondeuses pendant 4 semaines amena une augmentation progressive du résidu dans les oeufs, atteignant jusqu'à 2 ppm à la fin de la première semaine. Ce taux resta constant pendant le reste de l'expérience. Quand on sacrifice les poules pondeuses, on nota que le résidu trouvé dans leur tissu adipeux était légèrement plus important que chez les précédentes, mais il n'y avait pas de différence significative dans les tissus à consommer. Considérées à la lumière de toutes les données toxicologiques sur le BHT, ces résultats confirment l'inocuité du BHT ajouté à la nourriture des poules à un taux inférieur à 200 ppm.

## Rückstände von butyliertem Hydroxytoluol (BHT) und Stoffwechselprodukten in Geweben und Eiern von Hühnern, an die radioaktives BHT verfüttert wurde

**Zusammenfassung**—Radioaktives butyliertes Hydroxytoluol (BHT; 3,5-Di-*tert*-butyl-4-hydroxytoluol) mit $^{14}C$ im Ring wurde an eintägige Küken zehn Wochen lang in einer Konzentration von 200 ppm im Gesamtfutter verabreicht. Die Hühner wurden im Alter von 1, 2, 4 und 10 Wochen geschlachtet und alle Gewebe und Organe auf BHT und Stoffwechselprodukte analysiert. Maximaler Rückstandsgehalt wurde nach einer Woche festgestellt. Im grillreifen Alter enthielten die essbaren Teile 1 bis 3 ppm BHT und Stoffwechselprodukte. Etwas höhere Konzentrationen wurden im isolierten Fett, in der Haut und in den Verdauungsorganen festgestellt. Futter gleichen Gehalts, das Legenhennen 4 Wochen lang erhielten, führte zu allmählicher Rückstandszunahme in den Eiern bis zu einer Konzentration von 2 ppm nach 7 Tagen. Diese Konzentration blieb bis zum Ende des Versuchs konstant. Beim Schlachten war der Rückstandsgehalt im Fett der Legehennen etwas grösser als der in den Grillhühnern, aber die essbaren Gewebe unterschieden sich nicht wesentlich. Unter Berücksichtigung aller toxikologischen Daten über BHT bestätigen diese Ergebnisse, dass die Verabreichung von bis zu 200 ppm BHT im Hühnerfutter unbedenklich ist.

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# Progress Report on Multigeneration Reproduction Studies in Rats Fed Butylated Hydroxytoluene (BHT)

J. P. FRAWLEY

*Hercules Powder Company, Wilmington, Delaware, USA*

and

F. E. KOHN, J. H. KAY and J. C. CALANDRA

*Industrial Bio-Test Laboratories, Inc., Northbrook, Illinois, USA*

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Abstract—Weanling rats have been fed 20% fat diets containing 0, 300, 1000 or 3000 ppm butylated hydroxytoluene (BHT; 3,5-di-*tert*-butyl-4-hydroxytoluene). At 100 days of age, 16 females on each dietary level were mated with males on the same dietary level. Fertility, parturition and lactation were examined in the parents, and viability, survival, growth, and behaviour examined in the offspring. Ten days after weaning of the first litter, the females were mated a second time and the same examinations made on parents and offspring. Numerous function and clinical tests were performed on the parents after 28 wk on experimental diets and complete gross and microscopic pathology performed after weaning of the second litter, after 42 wk on test diets.

After weaning of the second litter, these offspring were fed the same diets as their parents until 100 days of age and mated in the same procedure. The same observations were made on these animals and 2 litters of offspring. A third generation is still under study.

At the 3000 ppm dietary level of BHT, a 10–20% reduction in growth rate of parents and offspring was observed. A 20% elevation of serum cholesterol levels was observed after 28 wk, but no cholesterol elevation after 10 wk. A 10–20% increase in relative liver weight was also observed in animals killed after 42 wk on diet. All other observations at 3000 ppm and all observations at 1000 and 300 ppm were comparable with those of the control group. All criteria of reproduction were normal. No teratogenic effects were detected.

By all criteria, in 2 generations of parent rats and their offspring, a "no-effect" level of 1000 ppm of BHT in a high fat diet is established.

## INTRODUCTION

Early investigations on the toxicology of butylated hydroxytoluene (BHT; 3,5-di-*tert*-butyl-4-hydroxytoluene) by Deichmann, Clemmer, Rakoczy & Bianchine (1955) failed to find any deleterious effect at dietary levels as high as 8000 ppm (0·8%) when fed for lifetime to rats. More recent investigations by Day, Johnson, O'Halloran & Schwartz (1959); [illegible], Johnson, & O'Halloran (1959); and Johnson & Hewgill (1961) employing diets containing 10–20% fat have clearly shown that slight liver and adrenal enlargement, [illegible] growth rate and elevated serum cholesterol occur at a 5000 ppm feeding level in [illegible] experiments. At 1000 ppm BHT, in high fat diets fed to rats for 6 wk, Johnson & [illegible] (1961) reported a slight elevation in serum cholesterol levels and adrenal weights. In [illegible] studies, Ames, Ludwig, Swanson & Harris (1956) reported no effect on reproduction of rats fed 0·31% in low fat diets. However, Brown *et al.* (1959) using high fat [illegible] reported occasional anophthalmia in offspring of rats fed 1000 and 5000 ppm BHT, [illegible] relate unequivocally this effect to the presence of BHT in the diet.

Since all previous reproduction experiments had been carried through one mating it was considered imperative to investigate the possible relationship of anophthalmia to BHT administration by conducting a multigeneration reproduction study with rats fed diets containing high levels of fat (20%). Moreover, the lack of confirmation by Day *et al.* (1959) of the effects on adrenal weight and serum cholesterol at the 1000 ppm level in paired feeding experiments and the lack of statistical verification of an effect at this level by Johnson & Hewgill (1961) indicated a need for more precise definition of a "no-effect" level. The experiments reported herein were designed to explore these questions in depth with large groups of test and control animals. Numerous additional parameters have been studied in an effort to uncover any possible deleterious effects from diets containing between 300 and 3000 ppm BHT.

## EXPERIMENTAL

*Animals.* Weanling (21-day-old) Sprague–Dawley albino rats were employed in these studies. A total of 80 males and 80 females was selected from a larger population of weanlings after examination of each animal for general physical well-being to form 3 experimental groups and 2 control groups designated the $F_0$ generation. Each group consisted of 16 males and 16 females.

*Diets.* The experimental diets were prepared by dissolving the BHT* in antioxidant-free lard which was specially prepared by Swift and Co., Chicago, Illinois, for these experiments. Weighed amounts of the lard solutions were incorporated into commercial ground laboratory diet† so that the final concentration of BHT in the 3 experimental diets were 300, 1000, and 3000 ppm. The animals on the control diets were fed the basic ration containing lard but no BHT. The total fat content of all diets was adjusted to 20% by the addition of appropriate amounts of lard. All lard and prepared diets were kept under constant refrigeration to prevent rancidity until distributed to the animals. Complete diets were made available to the rats every 7 days. Feeding was conducted *ad lib.* The appropriate diet was available to all animals at all times without interruption until sacrifice, including the mating and lactation periods of parental generations and pre-weaning period of the offspring.

*Mating procedure.* The initial groups of weanling rats ($F_0$ generation) were fed their respective diets until 100 days of age (79 days on test). Each of the 16 females in every group was caged randomly with a male from within the same group for 2 oestrous cycles. Daily observations for evidence of copulation (copulatory plug or bloody vagina) were made and recorded. The male was removed after evidence of copulation or after 2 oestrous cycles. If, after 2 oestrous cycles, the male failed to copulate, a second male was caged with the female for 2 oestrous cycles. Each female was given a maximum of 2 males for 2 oestrous cycles. Mating Index was calculated by dividing the number of copulations by the number of oestrous cycles × 100. The Fertility Index was calculated by dividing the number of actual pregnancies by the number of copulations × 100. Gestation times were recorded.

All offspring ($F_1$a generation) were examined on the day of birth for physical abnormalities and the number of viable and stillborn members of each litter was recorded. Records of survival at 1 and 5 days after birth were maintained and on the fifth day all litters of more than 10 offspring were reduced to that number by random sacrifice so that a more reliable index of lactation could be obtained. Observations for physical abnor-

*DalpacR R, Hercules Powder Company, Wilmington, Delaware, USA.
†Wayne Mouse Breeder Diet, Allied Mills, Inc., Chicago, Illinois, USA.

[illegible]ies continued until day 21 after birth, at which time the offspring were weaned and weighed.

After weaning of the $F_1a$ generation, the parental females ($F_0$) were given a 10-day rest period and again mated by the above procedure in order to obtain the $F_1b$ litters. After weaning of this second litter, the parental rats were maintained on experimental diet to permit clinical studies and were sacrificed after 42 wk on test for gross and microscopic pathology.

Sixteen female and 8 male 21-day-old $F_1b$ rats were selected randomly to represent the second parental generation. The animals were fed the same diet as their parents until 100 days of age and mated in the same procedure to yield 2 litters $F_2a$ and $F_2b$. The second litters ($F_2b$) were selected as the parental animals for the third generation.

*Observations.* Initially the body weight of each $F_0$ generation rat was determined and recorded. Thereafter, all animals were weighed weekly until mating trials commenced. No further body weight data were obtained until sacrifice of the $F_0$ generation because of large variations due to pregnancies. All offspring were weighed at weaning (21 days postpartum). The same weighing schedule was followed for all generations.

After 10 wk on the test diet and again after weaning of the second litter (28 wk), 10 male and 10 female rats from the $F_0$ parental groups, including controls, were bled for total serum cholesterol concentration (Caraway & Fanger, 1955). This procedure was repeated on the other parental groups on all 8 males and 10 females after weaning of the second litters.

In addition the following determinations were made after 28 wk on 5 male and 5 female rats of each parental generation from the control groups and the 3000 ppm BHT group. Complete blood counts (haemoglobin, erythrocyte, total and differential leukocyte), cell indices (mean corpuscular volume of haemoglobin concentration and colour saturation and volume indices) reticulocyte count, prothrombin time, serum glutamic–pyruvic transaminase and serum glutamic–oxaloacetic transaminase (Reitman & Frankel, 1957), fasted blood sugar, serum alkaline phosphatase (Bessey, Lowry & Brock, 1946), blood urea nitrogen and icterus index. Urine analyses at the same time included measurement of reducing substances, albumin and microscopic elements.

Following completion of the clinical studies and 42 wk of consumption of the test diets, the parental animals were sacrificed for gross and microscopic pathology. Organ weights obtained included liver, kidney, spleen, gonads, heart, brain, thyroid, and adrenal. Organ weight data were treated for statistical significance by an analysis of variance followed by Student's *t* test. Microscopic pathologic studies included: heart (right and left ventricles), trachea, lung, liver, pancreas, stomach (cardiac, fundic and pyloric regions), small intestine (duodenum, jejunum, ileum), caecum, colon, spleen, lymph node (cervical and mesenteric), kidney, urinary bladder, testis, ovary, prostate, uterus, pituitary gland, salivary gland (submaxillary), thyroid gland, parathyroid gland, adrenal gland, skeletal muscle, bone marrow (sternum and femur), peripheral nerve (sciatic), brain (cerebrum, cerebellum, pons), seminal vesicles, oesophagus, and spinal cord. These examinations were performed on each parental generation.

## RESULTS

### *Parent generation*

*Animal behaviour and appearance.* The only adverse reactions observed in any of the animals was occasional slight corneal opacity in both control and test animals. The incidence was not significantly different for any test group.

*Body weight.* Body weight gains for the parental animals after 10-wk feeding of test and control diets are presented in Table 1. Approximately a 10% reduction in weight gain was observed in the male rats only, which were fed 3000 ppm BHT. This effect was of equal magnitude for both generations. No effect on growth rate was observed at 1000 ppm.

Table 1. *Mean body weights of parent generations ($F_0$ and $F_1b$) after 10 wk on BHT diets and prior to first mating*

| Dietary level of BHT (ppm) | Body weight (g) | | | |
|---|---|---|---|---|
| | Male | | Female | |
| | $F_0$ | $F_1b$ | $F_0$ | $F_1b$ |
| 0 (Control 1) | 288 | 292 | 177 | 170 |
| 0 (Control 2) | 305 | 311 | 192 | 183 |
| 300 | 307 | 286 | 180 | 175 |
| 1000 | 291 | 296 | 189 | 176 |
| 3000 | 266* | 268* | 180 | 168 |

*Statistically-significant effect.

*Cholesterol levels.* Table 2 provides the mean serum cholesterol levels at 10 and 28 wk for male and female parents of the first 2 generations. These mean values are the result of duplicate analyses on 10 individual animals. Considerable individual variation existed between the animals on the same diet. For example, the range of cholesterol levels on the males of control group 1 was 84–142 mg/100 ml at 10 wk and 80–120 mg/100 ml at 28 wk. Statistical examination of these data revealed no significant difference of serum cholesterol at 10 wk for any of the feeding levels. However, at 28 wk, a significant elevation was detected in the male and female rats fed 3000 ppm BHT, but not in those fed 1000 ppm or 300 ppm.

Table 2. *Mean serum cholesterol concentrations of parent generations ($F_0$ and $F_1b$) on BHT diets before mating and after production of two litters*

| Dietary level of BHT (ppm) | Cholesterol level (mg/100 ml) at wk | | | | | |
|---|---|---|---|---|---|---|
| | 10 | | 28 | | | |
| | Male | Female | Male | | Female | |
| | $F_0$ | $F_0$ | $F_0$ | $F_1b$ | $F_0$ | $F_1b$ |
| 0 (Control 1) | 116 | 106 | 95 | 93 | 87 | 108 |
| 0 (Control 2) | 109 | 116 | 99 | 99 | 91 | 124 |
| 300 | 111 | 121 | 90 | 93 | 97 | 94 |
| 1000 | 116 | 101 | 111 | 113 | 116 | 125 |
| 3000 | 125 | 107 | 128* | 127* | 123* | 149* |

*Statistically-significant effect.

*Clinical studies.* No differences between test and control animals were revealed by the haematology or urine studies, nor by any of the blood chemistry or function tests performed.

*Mortality.* No significant mortality was observed in either of the control or test parental generations. Pathological examination on all dead animals revealed severe pneumonitis as

cause for the few deaths which occurred, with the exception of one control, which was attributed to uterine haemorrhage at parturition.

*Organ weights.* Table 3 reports the mean relative liver weights of the male and female parent generations for all control and test groups sacrificed after weaning of the second litters. Statistically significant enlargement of the liver was observed at the 3000 ppm BHT feeding level for both males and females. Examination of the absolute liver weights and calculation of the liver to brain weight ratio failed to demonstrate a significant enlargement in the males at 3000 ppm, indicating that the decreased final body weight contributed partly to the significance of the elevation of the liver to body weight ratio. No consistent effect on absolute liver weights and liver weight to body weight ratios was demonstrated by statistical examination at the 1000 or 300 ppm level.

Table 3. *Mean relative liver weights* of parent generations ($F_0$ and $F_1b$) on BHT diets after production of two litters*

| Dietary level of BHT (ppm) | Relative liver weight (%) | | | |
|---|---|---|---|---|
| | Male | | Female | |
| | $F_0$ | $F_1b$ | $F_0$ | $F_1b$ |
| 0 (Control 1) | 2·71 | 2·86 | 2·67 | 3·01 |
| 0 (Control 2) | 2·87 | 3·17 | 2·55 | 2·99 |
| 300 | 3·07 | 3·18 | 2·73 | 2·98 |
| 1000 | 3·12 | 3·08 | 2·79 | 3·08 |
| 3000 | 3·42† | 3·65† | 3·22 | 3·67† |

*Ratio of liver weight to body weight (%).
†Statistically-significant effect.

No statistically-significant effects on the weight of any other organ was observed at any of the feeding levels. Of particular note was the absence of any effect on relative adrenal weights (Table 4) which had been reported by Johnson & Hewgill (1961) to be enlarged even at the 1000 ppm level.

Table 4. *Mean relative adrenal weights* of parent generations ($F_0$ and $F_1b$) on BHT diets after production of two litters*

| Dietary level of BHT (ppm) | Relative adrenal weight (mg/100 g) | | | |
|---|---|---|---|---|
| | Male | | Female | |
| | $F_0$ | $F_1b$ | $F_0$ | $F_1b$ |
| 0 (Control 1) | 8·6 | 11 | 17 | 23 |
| 0 (Control 2) | 9·0 | 11 | 17 | 23 |
| 300 | 8·0 | 14 | 16 | 27 |
| 1000 | 8·6 | 13 | 17 | 24 |
| 3000 | 8·5 | 11 | 16 | 25 |

*Ratio of adrenal weight to body weight (mg/100g).

*Gross and microscopic pathology.* Both gross and microscopic pathological studies revealed no tissue changes which could be attributed to ingestion of BHT for 42 wk by either generation. Usual incidence of slight to mild histopathological changes were observed

in control and test animals, as lung hyperaemia and pneumonitis, chronic focal inflammation of small and large intestine, kidney nephrosis, etc. Only one tumour was found in any of the animals, a subcutaneous fibroadenoma in the right axillary region of a female rat fed 3000 ppm BHT.

*Mating performance.* Table 5 provides the Mating Index for the 2 parent generations of the 5 groups of animals and reveals no interference with desire or ability to copulate from any level of BHT. Table 6 provides the Fertility Index for the same animals and reveals no effect on fertility or ability to conceive. Table 7 provides the Parturition Index and reveals no effect on sustaining the pregnancies to parturition. Mean gestation time for all groups was either 21 or 22 days. Table 8 provides the Lactation Index demonstrating no interference with lactation and nursing of the offspring.

**Table 5.** *Mating Index* of parent generations ($F_0$ and $F_1b$) on BHT diets*

| Dietary level of BHT (ppm) | Mating Index (%) | | | |
|---|---|---|---|---|
| | $F_0$ | | $F_1b$ | |
| | $F_1a$ | $F_1b$ | $F_2a$ | $F_2b$ |
| 0 (Control 1) | 39 | 57 | 49 | 90 |
| 0 (Control 2) | 48 | 74 | 53 | 85 |
| 300 | 63 | 74 | 46 | 78 |
| 1000 | 34 | 62 | 49 | 57 |
| 3000 | 44 | 81 | 52 | 84 |

*Mating Index is the ratio of no. of successful copulations to no. of mating opportunities × 100.

**Table 6.** *Fertility Index* of parent generations ($F_0$ and $F_1b$) on BHT diets*

| Dietary level of BHT (ppm) | Fertility Index (%) | | | |
|---|---|---|---|---|
| | $F_0$ | | $F_1b$ | |
| | $F_1a$ | $F_1b$ | $F_2a$ | $F_2b$ |
| 0 (Control 1) | 93 | 94 | 94 | 89 |
| 0 (Control 2) | 100 | 94 | 100 | 88 |
| 300 | 100 | 94 | 84 | 89 |
| 1000 | 92 | 100 | 65 | 88 |
| 3000 | 93 | 100 | 100 | 100 |

*Fertility Index is the ratio of no. of pregnancies to no. of successful copulations × 100.

Table 7. *Parturition Index* of parent generations ($F_0$ and $F_1b$) on BHT diets*

| Dietary level of BHT (ppm) | Parturition Index (%) | | | |
|---|---|---|---|---|
| | $F_0$ | | $F_1b$ | |
| | $F_1a$ | $F_1b$ | $F_2a$ | $F_2b$ |
| 0 (Control 1) | 100 | 100 | 100 | 100 |
| 0 (Control 2) | 100 | 94 | 100 | 100 |
| 300 | 100 | 94 | 100 | 100 |
| 1000 | 91 | 100 | 100 | 100 |
| 3000 | 93 | 100 | 100 | 94 |

*Parturition Index is the ratio of no. of parturitions to no. of pregnancies ×100.

Table 8. *Lactation Index* of parent generations ($F_0$ and $F_1b$) on BHT diets*

| Dietary level of BHT (ppm) | Lactation Index (%) | | | |
|---|---|---|---|---|
| | $F_0$ | | $F_1b$ | |
| | $F_1a$ | $F_1b$ | $F_2a$ | $F_2b$ |
| 0 (Control 1) | 94 | 99 | 80 | 72 |
| 0 (Control 2) | 90 | 97 | 94 | 88 |
| 300 | 95 | 94 | 86 | 88 |
| 1000 | 97 | 95 | 92 | 61 |
| 3000 | 99 | 95 | 69 | 75 |

*Lactation Index is the ratio of no. of pups viable at 21 days to no. viable at 5 days ×100.

*Offspring*

*Body weight.* Table 9 lists the mean litter size (number of viable offspring per litter on day of birth) for all groups of animals and demonstrates normal distribution. Table 10 provides the mean body weight of these litters at 21 days of age. A significant depression in body weight is noted at the 3000 ppm BHT feeding level, as previously observed with both parent generations. Even though these animals were smaller than the controls, presumably due to pre-weaning ingestion of the BHT diet, growth rate of the $F_1b$ litter for 10 wk after weaning was comparable to the original parental generation (as shown in Table 1). No effect on weaning weight was detected at 1000 or 300 ppm BHT.

Table 9. *Mean litter size of progeny of parent generations ($F_0$ and $F_1b$) on BHT diets*

| Dietary level of BHT (ppm) | Mean no. of viable pups per litter | | | |
|---|---|---|---|---|
| | $F_0$ | | $F_1b$ | |
| | $F_1a$ | $F_1b$ | $F_2a$ | $F_2b$ |
| 0 (Control 1) | 11·5 | 13·2 | 8·9 | 10·9 |
| 0 (Control 2) | 11·9 | 10·2 | 11·1 | 13·0 |
| 300 | 11·5 | 10·6 | 11·9 | 10·3 |
| 1000 | 10·0 | 13·0 | 10·8 | 11·3 |
| 3000 | 10·6 | 10·1 | 9·3 | 10·9 |

Table 10. *Mean body weight of weanlings of parent generations ($F_0$ and $F_1b$) on BHT diets*

| Dietary level of BHT (ppm) | Mean body weight (g) at 21 days | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Male | | | | Female | | | |
| | $F_0$ | | $F_1b$ | | $F_0$ | | $F_1b$ | |
| | $F_1a$ | $F_1b$ | $F_2a$ | $F_2b$ | $F_1a$ | $F_1b$ | $F_2a$ | $F_2b$ |
| 0 (Control 1) | 55 | 52 | 44 | 45 | 52 | 51 | 43 | 44 |
| 0 (Control 2) | 51 | 56 | 47 | 48 | 50 | 53 | 44 | 43 |
| 300 | 53 | 54 | 45 | 46 | 51 | 52 | 43 | 45 |
| 1000 | 54 | 53 | 44 | 50 | 54 | 49 | 41 | 46 |
| 3000 | 41* | 45* | 41 | 39* | 38* | 43* | 40 | 40 |

*Statistically-significant effect.

*Mortality.* Table 11 provides the Viability Index for both litters of both parent generations. No significant number of offspring were born dead in any of the groups. The Survival Index through the first 5 days is shown in Table 12, demonstrating normal survival at all levels. The Lactation Index (Table 8) also reveals normal survival until weaning on day 21.

Table 11. *Viability Index* of progeny of parent generations ($F_0$ and $F_1b$) on BHT diets*

| Dietary level of BHT (ppm) | Viability Index at birth (%) | | | |
|---|---|---|---|---|
| | $F_0$ | | $F_1b$ | |
| | $F_1a$ | $F_1b$ | $F_2a$ | $F_2b$ |
| 0 (Control 1) | 99 | 100 | 90 | 94 |
| 0 (Control 2) | 98 | 88 | 96 | 99 |
| 300 | 100 | 98 | 99 | 88 |
| 1000 | 87 | 99 | 100 | 99 |
| 3000 | 100 | 97 | 92 | 98 |

*Viability Index is the ratio of no. of viable pups to total born × 100.

Table 12. *Survival Index* of progeny of parent generations ($F_0$ and $F_1b$)*

| Dietary level of BHT (ppm) | Survival Index (%) | | | |
|---|---|---|---|---|
| | $F_0$ | | $F_1b$ | |
| | $F_1a$ | $F_1b$ | $F_2a$ | $F_2b$ |
| 0 (Control 1) | 96 | 94 | 99 | 90 |
| 0 (Control 2) | 95 | 94 | 97 | 91 |
| 300 | 96 | 94 | 94 | 90 |
| 1000 | 87 | 91 | 92 | 88 |
| 3000 | 93 | 96 | 86 | 85 |

*Survival Index is the ratio of no. of pups viable at day 5 to no. of viable pups born × 100.

*Teratogenic effects.* No untoward reactions or physical abnormalities w
of the offspring. No evidence of anophthalmia or any other teratog
detected.

## CONCLUSIONS

A multigeneration reproduction study with rats continuously fed 300
BHT in diets containing 20% fat has been conducted. Data are re
parent generations and two litters of offspring for each generation. No ter
have been observed in any of the 4 litters at any feeding level. The only si
observed were a 10–20% reduction in growth rate of parents and offspring,
of serum cholesterol and a 20% increase in the relative liver weight of par
ppm feeding level. The data fail to confirm previous reports of adrenal
3000 and 1000 ppm, and also fail to confirm an elevation of cholesterol or
at 1000 ppm. By all criteria, a "no-effect" level of 1000 ppm BHT is estal
generation is also under study.

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## Rapport sur les travaux en cours, concernant la reproduction du rat, à travers plusieurs générations d'animaux ayant absorbé du but d'hydroxytoluène (BHT) dans leurs aliments

Résumé—Des rats nouvellement sevrés ont reçu une ration alimentaire à 20%
contenant 0, 300, 1000 et 3000 ppm de butylate d'hydroxytoluène (BHT; 3,5-di
hydroxytoluène). A l'âge de 100 jours, on a accouplé 16 femelles de chacune de c
avec des mâles de la même catégorie. On a étudié la fertilité, la parturition et la l
les parents, ainsi que la viabilité, la longueur de vie, la croissance et le comporte
jeunes. Dix jours après le sevrage de la première portée, les femelles ont été ac
seconde fois et les mêmes examens ont été répétés sur les parents et sur les
pratiqué de nombreux tests fonctionnels et cliniques sur les parents après 28

régime, de même qu'une étude anatomo-pathologique macroscopique et microscopique complète après le sevrage de la seconde portée, au bout de 42 semaines de régime.

Après le sevrage de la seconde portée, les petits qui la composaient ont été mis au même régime que leurs parents jusqu'à l'âge de 100 jours et accouplés de la même façon. Les mêmes observations ont été conduites sur ces animaux et sur deux portées de petits. Une troisième génération est en cours d'étude.

Pour un taux de BHT de 3000 ppm dans le régime alimentaire, on a observé un abaissement de la courbe de croissance chez les parents et chez les enfants. Une élévation du taux du cholestérol sérique de 20% a été notée au bout de 28 semaines, tandis qu'aucune modification n'était apparente après 10 semaines. On a observé aussi une augmentation de 10 à 20% du poids relatif du foie chez les animaux sacrifiés au bout de 42 semaines de régime. Toutes les autres observations faites sur les animaux qui avaient reçu 3000 ppm du produit ainsi que sur ceux qui en avaient reçu 1000 et 300 ppm ont été comparables à celles du groupe témoin. Tous les critères de reproduction ont été normaux. Aucun effet tératogène n'a été détecté.

Tous les résultats des examens pratiqués sur deux générations de rats, parents et descendance, concordent pour prouver qu'un taux de 1000 ppm de BHT dans un régime alimentaire riche en lipides n'a pas d'effet nocif.

## Arbeitsbericht über die sich auf mehrere Generationen erstreckenden Fortpflanzungsuntersuchungen an mit butyliertem Hydroxytoluol (BHT) gefütterten Ratten

**Zussmmenfassung**—Abgesäugte junge Ratten erhielten Futter mit 20% Fettgehalt und 0, 300, 1000 und 3000 ppm butyliertem Hydroxytoluol (BHT; 3,5-Di-*tert*-butyl-4-hydroxytoluol). Im Alter von 100 Tagen wurden 16 weibliche Tiere jeder der Versuchsdiätgruppen mit männlichen Tieren der korrespondierenden Gruppen gepaart. Fertilität, Geburtsvorgang und Laktation bei den Eltern und Lebensfähigkeit, Überlebensdauer, Wachstum und Verhalten bei den Nachkommen wurden beobachtet. Zahlreiche Funktions-und klinische Untersuchungen wurden an den Eltern nach 26 wöchiger Versuchsdiät und vollständige makroskopische und mikroskopische pathologische Untersuchungen wurden nach dem Absetzen der zweiten Nachkommengeneration nach 42 Wochen Versuchsdiät vorgenommen.

Nach dem Absetzen der zweiten Nachkommengeneration erhielt diese bis zum Alter von 100 Tagen die gleiche Versuchsdiät wie die Elterngeneration. Dann wurde sie in der gleichen Weise gepaart. Die gleichen Beobachtungen wurden an diesen Tieren und 2 Generationen Nachkommen durchgeführt. Eine dritte Generation befindet sich noch unter Beobachtung.

Bei der Verfütterung von 3000 ppm BHT wurde eine 10- bis 20% ige Verminderung der Wachstumsgeschwindigkeit bei Eltern und Nachkommen festgestellt. Nach 28 Wochen wurde eine 20% ige Erhöhung des Serumcholesterinspiegels beobachtet, aber keine Cholesterinerhöhung nach 10 Wochen. Das Lebergewicht der nach 42 Wochen Versuchsdiät getöteten Tiere war 10 bis 20% erhöht. Alle anderen Beobachtungen an der 3000-ppm-Gruppe und alle Beobachtungen an den 1000- und 300-ppm-Gruppen waren mit denen an der Kontrollgruppe vergleichbar. Alle Fortpflanzungskriterien waren normal. Es wurden keine teratogenen Wirkungen festgestellt.

Bezüglich aller Kriterien wurde in 2 Generationen von Eltern und Nachkommen bei Ratten die Unschädlichkeit von 1000 ppm BHT in Futter hohen Fettgehalts konstatiert.

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# Liver Response Tests. IV. Application to Short-term Feeding Studies with Butylated Hydroxytoluene (BHT) and Butylated Hydroxyanisole (BHA)*

J. F. GAUNT, G. FEUER, F. A. FAIRWEATHER† and D. GILBERT

*British Industrial Biological Research Association, Woodmansterne Road, Carshalton, Surrey, England*

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Abstract—Preliminary studies involved oral administration to rats of 6 daily doses (500 mg/kg) of butylated hydroxytoluene (BHT; 3,5-di-*tert*-butyl-4-hydroxytoluene) or butylated hydroxyanisole (BHA; 3-*tert*-butyl-4-hydroxyanisole). Both BHA and BHT induced liver enlargement and increased daily output of urinary ascorbic acid. These changes paralleled each other closely in onset, degree and duration, being rapid but transient with BHA, and slower in onset yet more prolonged with BHT.

Administration of BHA or BHT at a level of 0·1% in the diet of rats for 16 wk elicited similar changes, particularly in females. With BHT the effects were more pronounced and more prolonged, but despite continuation of treatment relative liver weight and urinary ascorbic acid had returned to control levels by 16 wk. In the case of BHA the effects were no longer manifest after 4 wk. There was no histopathological evidence of damage to the liver. Equally negative findings regarding liver response were obtained in the biochemical measurements and histochemical assessments of glucose 6-phosphatase and glucose 6-phosphate dehydrogenase activities. Both BHT and BHA brought about increases in adrenal weight.

It is concluded that measurement of ascorbic acid excretion in the urine affords a promising means of detecting hyperfunctional liver enlargement and distinguishing it from toxic liver enlargement. Under the conditions of the present experiments neither BHT nor BHA exhibited evidence of hepatotoxicity.

## INTRODUCTION

The preceding communication in this series (Gilbert & Golberg, 1965) confirmed the findings of earlier workers (Deichmann, Clemmer, Rakoczy & Bianchine, 1955; Brown, Johnson & O'Halloran, 1959; Johnson & Hewgill, 1961) concerning the capacity of high doses of butylated hydroxyanisole (BHA; 3-*tert*-butyl-4-hydroxyanisole) or butylated hydroxytoluene (BHT; 3,5-di-*tert*-butyl-4-hydroxytoluene) to induce an increase in relative liver weight in rats. It was established that, at least in the case of BHT, liver enlargement reflected a hyperfunctional state related to increased activity of microsomal processing enzymes of the liver. The criteria used to demonstrate this change involved biochemical measurement of the activities of the enzymes hexobarbitone oxidase, *o*-nitroanisole demethylase and antipyrine demethylase in liver homogenates.

The studies to be described here were intended to investigate the effects of feeding relatively high levels of BHA or BHT (0·1%) in the diet of rats over a 4-month period. In the course of the experiment the following aspects were examined: the value of measuring 24-hr urinary output of ascorbic acid as an index of hyperfunctional liver enlargement; the liver response, in terms of the activities of glucose 6-phosphatase and glucose 6-phosphate

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† Present address: John Wyeth & Brother, Huntercombe Lane South, Taplow, Maidenhead, Berkshire.

dehydrogenase assessed biochemically (Feuer, Golberg & Le Pelley, 1965a,b) as well as histochemically; and the usual criteria employed in the study of short-term toxicity.

## EXPERIMENTAL

*Materials.* Samples of BHT and BHA were obtained from the same sources and conformed to the same specifications as described by Gilbert & Golberg (1965).

### *Preliminary studies—ascorbic acid excretion in urine*

*Animals and dosage.* Preliminary studies were carried out on groups of 8 female Porton SPF albino rats weighing 150–205 g, caged in pairs. The experiments involved daily administration by stomach tube of BHA or BHT in doses of 500 mg/kg dissolved in arachis oil (5 ml/kg). Control groups of equal size were given arachis oil. It was found that, on transferring the animals to metabolism cages, a period of 5–6 days was necessary for the urinary ascorbic acid output to reach a basal level.

The studies with BHA were repeated using groups of 10 male (body weight 155–160 g) and 10 female (body weight 125–130 g) Carworth Farm SPF rats which received the same treatment as above. Appropriate control groups were also set up.

*Determination of ascorbic acid in urine and adrenals.* The daily excretion of ascorbic acid in the urine was determined by the method of Harris & Olliver (1942) in the course of the experiments with BHA and BHT using Porton rats. For the study of BHA in Carworth rats the method of Howard & Constable (1965) was modified to use 2 titrations with 2,6-dichlorophenolindophenol, thus replacing the colorimetric estimation of an added excess of the reagent. The latter method was also employed for the urinary measurements made in the course of the 16-wk study and for the determination of adrenal ascorbic acid in preliminary studies in Carworth rats. The adrenals were homogenized in trichloroacetic acid [2 ml of 4% (w/v)] in acetone [10% (w/v)].

### *16-Wk study*

*Animals and dosage.* Three groups of 48 weanling Carworth SPF rats (24 males and 24 females), housed in cages of 6, were maintained on Spiller's Small Laboratory Animal diet, reground to a powder, and water *ad lib.* These groups received basal diet (control) or dietary levels of 0·1% BHA or 0·1% BHT for periods of up to 16 wk.

*Conduct of experiment.* Rats were examined twice daily for general condition. Weekly records were kept of individual body weights and of the food consumption of each cage of rats. Cages of 6 rats from each group were killed at 4, 6, 12 and 16 wk. Daily urine samples were collected in trichloroacetic acid [2 ml of 40% (w/v)] for 4 days before killing for the determination of ascorbic acid excretion.

At autopsy the major organs (brain, heart, liver, spleen, kidneys, adrenals and testes or ovaries) were weighed and samples of the right lobe of the liver fixed in buffered formalin (4% formaldehyde BP in 0·058 M-phosphate buffer, pH 7·0) for histological examination. Histological, histochemical and biochemical examinations were performed on the liver of each rat, except at wk 12 when histochemical studies were not carried out.

*Histochemical examination of the liver.* Histochemical examination was carried out on frozen liver sections for the following enzymes: acid phosphatase (Gomori, 1950), glucose 6-phosphatase (Wachstein & Meisel, 1956) and glucose 6-phosphate dehydrogenase (Pearse, 1961).

*Biochemical examination of the liver.* Assays of liver glucose 6-phosphatase and glucose 6-phosphate dehydrogenase were carried out using the methods previously described by Feuer *et al.* (1965a).

## RESULTS

*Preliminary measurements of urinary ascorbic acid excretion*

The administration of 6 oral doses of BHA or BHT (500 mg/kg/day) to female Porton rats produced effects on urinary ascorbic acid and on relative liver weight which are shown in Fig. 1. It is clear that the ascorbic acid output remained at a low level in control animals given arachis oil only. With BHA there was a sharp rise in ascorbic acid excretion, reaching a peak at 24–48 hr and thereafter declining to levels only slightly higher than those of

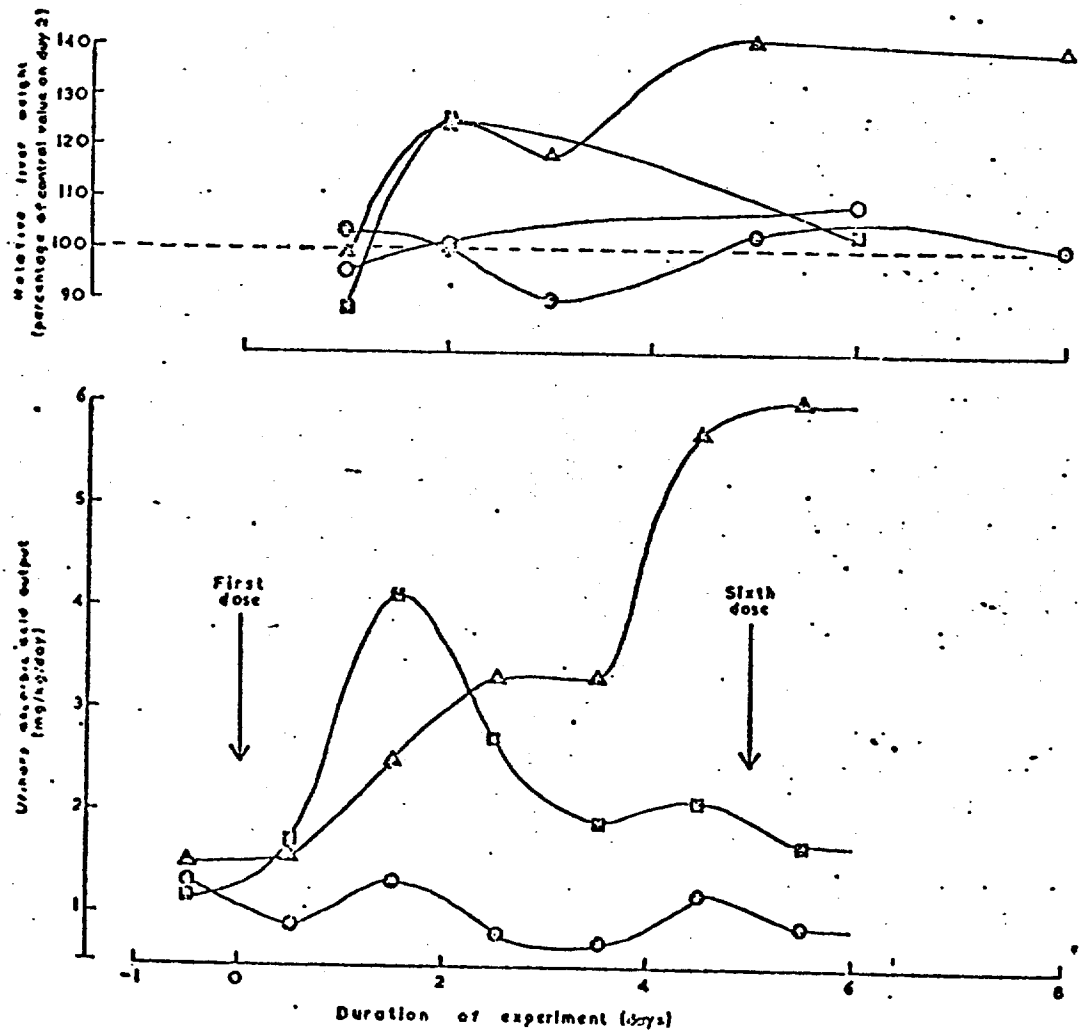


Fig. 1. Changes in urinary ascorbic acid and relative liver weight in female rats. BHT (△) or BHA (□) administered at 500 mg/kg/day by stomach tube for 6 days. Controls (⊙) for BHT or (O) for BHA received arachis oil by the same route. Points on the ascorbic acid curves are located midway between each day and represent the total output during the 24-hr period after each dose.

controls. The transient increase in ascorbic acid output was reflected in an almost exact contemporaneous increase in relative liver weight; the hepatomegaly was equally transient as judged by the liver weights of the group killed at 6 days, which were within the normal range.

Administration of BHT elicited a more gradual rise in urinary ascorbic acid, reaching a high plateau on about the fifth day. The rise in liver weight proved to be more sustained than in the case of BHA and the high degree of enlargement attained by the liver on the fifth day was maintained until the eighth day, despite the discontinuance of BHT administration.

Later, the experiment with BHA was repeated, using both male and female rats of the Carworth strain. The results (Fig. 2) again show a rise in ascorbic acid excretion in females having the same time relationships and other characteristics described above. The males, on the other hand, failed to show an effect in comparison with controls. The terminal relative liver weights (as percentage of body weight), measured 24 hr after the tenth daily dose of BHA had been given, were: control males 5·62±0·21; BHA males 6·22±0·19 ($P$ <0·01); control females 4·73±0·13; BHA females 5·55±0·08 ($P$ <0·01). Adrenal ascorbic acid contents of pooled specimens from the 4 groups were, respectively, 1·09, 2·75, 2·45 and 2·34 mg/g.

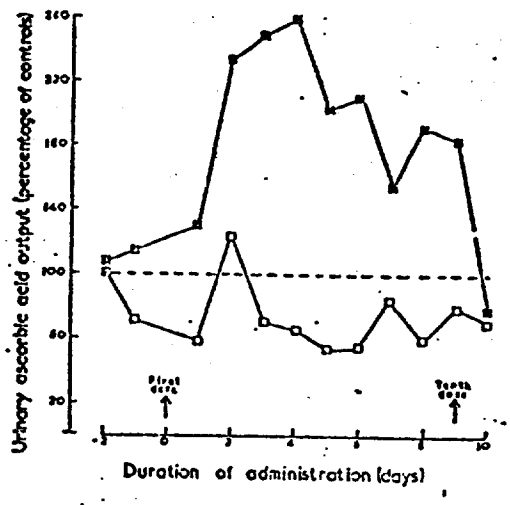


FIG. 2. 24-Hr urinary ascorbic acid output in male (■) and female (□) rats given BHA. Carworth rats were given 10 consecutive daily doses of BHA (500 mg/kg) by stomach tube. Controls received arachis oil and results are expressed as percentage of control values determined on the same day. Points on the ascorbic acid curves are located midway between each day and represent the total output during the 24-hr period after each dose.

### 16-*Wk study*

Both BHA and BHT at levels of 0·1% of the diet caused significant increases in urinary ascorbic acid excretion which were observed in females at 4, 6 and 16 wk and in males at 4 and 12 wk following BHT treatment and in females at 4 wk following BHA treatment (Table 1). The scatter of individual values tended to mask possibly enhanced values at other times.

The growth of animals on BHT or BHA closely paralleled those of the controls, except towards the end of the 16-wk period in males receiving BHA, where there was a retardation

Table 1. *Urinary ascorbic acid excretion and relative liver and adrenal weights of rats fed BHA or BHT at 0·1% of the diet for 16 wk*

| Compound | Duration of feeding (wk) | Ascorbic acid (mg/kg/day) | | Organ weight to body weight ratio (%) | | | |
|---|---|---|---|---|---|---|---|
| | | | | Liver | | Adrenal | |
| | | Males | Females | Males | Females | Males | Females |
| Arachis oil, control | 4 | 2·20±0·39 | 3·25±0·35 | 4·21±0·11 | 3·94±0·08 | 0·010±0·0008 | 0·016±0·0005 |
| BHT | | 4·74±0·95* | 7·83±1·27** | 4·68±0·16** | 4·45±0·08*** | 0·013±0·0010* | 0·018±0·0008 |
| BHA | | 1·84±0·24 | 4·16±0·38* | 4·36±0·15 | 4·50±0·12** | 0·012±0·0007 | 0·018±0·0011 |
| Arachis oil, control | 6 | 4·39±1·08 | 1·28±0·38 | 4·13±0·05 | 3·71±0·11 | 0·010±0·0005 | 0·018±0·0011 |
| BHT | | 6·90±1·76 | 3·39±0·51* | 4·18±0·06 | 4·00±0·09* | 0·010±0·0004 | 0·015±0·0009 |
| BHA | | 4·89±1·56 | 1·67±0·51 | 4·24±0·12 | 3·82±0·12 | 0·010±0·0011 | 0·017±0·0006 |
| Arachis oil, control | 12 | 2·10±0·22 | 3·12±0·57 | 3·73±0·07 | 3·15±0·10 | 0·009±0·0037 | 0·017±0·0008 |
| BHT | | 3·41±0·41* | 4·83±0·63 | 3·93±0·07* | 3·51±0·08* | 0·010±0·0026 | 0·022±0·0005** |
| BHA | | 1·87±0·37 | 2·32±0·36 | 3·58±0·14 | 3·60±0·06** | 0·011±0·0008 | 0·020±0·0009* |
| Arachis oil, control | 16 | 2·50±0·34 | 0·98±0·14 | 3·50±0·10 | 3·21±0·10 | 0·009±·00081 | 0·016±0·0014 |
| BHT | | 3·13±0·43 | 2·37±0·37** | 3·73±0·08 | 3·63±0·10* | 0·010±0·0061 | 0·019±0·0009 |
| BHA | | 2·29±0·24 | 0·92±0·17 | 3·72±0·09 | 3·40±0·17 | 0·011±0·0005 | 0·020±0·0004* |

Each result represents the mean ±SE for 6 animals; ascorbic acid is the mean of 4 days' excretion. Figures marked with asterisks differ significantly from those of controls: * $P<0·05$; ** $P<0·01$; *** $P<0·001$.

of growth (Fig. 3) together with a decrease in food consumption (Fig. 4). It must be [illegible] however, that only 6 animals remained at this stage and that they were transferred [illegible] metabolism cages for urine collection.

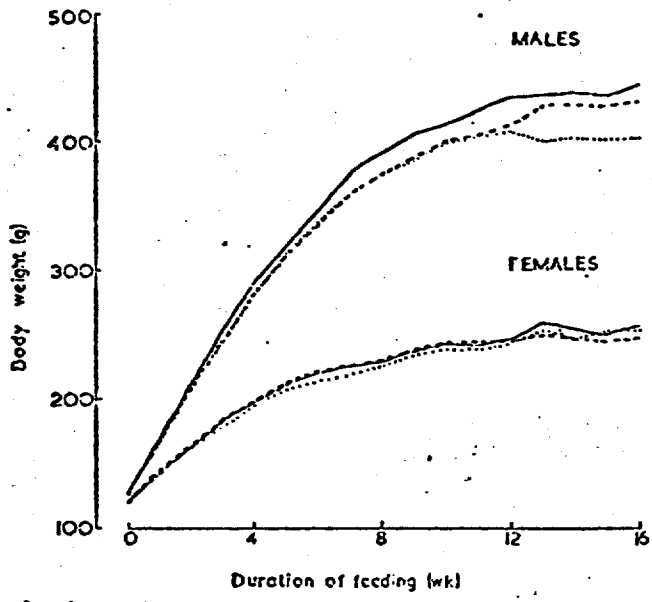


FIG. 3. Growth rate of male and female rats fed on basal diet (——); 0·1% BHT (– – –) or 0·1% BHA (· · · · ·) for periods up to 16 wk. Lots of 6 rats were removed from each group for examination at wk 6, 12 and 16.

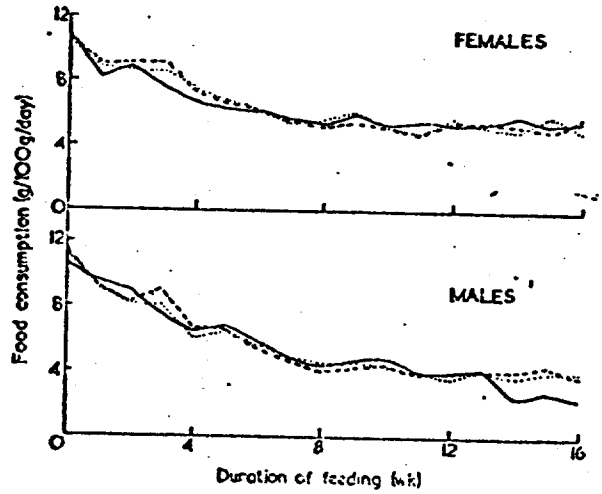


FIG. 4. Food consumption in male and female rats fed on basal diet (——); 0·1% BHT (– – –) or 0·1% BHA (· · · · ·) for periods of up to 16 wk. Lots of 6 rats were removed from each group for examination at wk 4, 6, 12 and 16.

Changes in organ weight at autopsy were mainly confined to the liver and adrenals (Table 1). Increases in relative liver weight occurred predominantly in females given BHT or BHA. In a few instances adrenal weights were also raised by both compounds, again mainly in females.

Biochemical tests of liver response were applied at intervals during the course of the experiment (Table 2). After 4 or 6 wk on diets containing BHA or BHT there was no change in glucose 6-phosphatase activity; after 12 wk the activity of this enzyme fell below the normal level in both sexes receiving BHT, but was found to be the same as in controls at 16 wk. The only change in glucose 6-phosphate dehydrogenase activity was an increase seen at 4 wk in females given BHA.

Table 2. *Liver glucose 6-phosphatase and glucose 6-phosphate dehydrogenase activities of rats fed BHT or BHA at 0·1% of the diet for 16 wk*

| Compound | Duration of feeding (wk) | Glucose 6-phosphatase | | Glucose 6-phosphate dehydrogenase | |
|---|---|---|---|---|---|
| | | Males | Females | Males | Females |
| Arachis oil, control | 4 | 1·73±0·08 | 1·57±0·07 | 0·489±0·063 | 0·534±0·042 |
| BHT | | 1·82±0·09 | 1·50±0·07 | 0·516±0·039 | 0·564±0·039 |
| BHA | | 1·82±0·07 | 1·59±0·03 | 0·459±0·033 | 0·726±0·045** |
| Arachis oil, control | 6 | 1·93±0·08 | 1·63±0·05 | 0·402±0·018 | 0·546±0·036 |
| BHT | | 1·72±0·13 | 1·73±0·06 | 0·390±0·030 | 0·474±0·063 |
| BHA | | 1·67±0·12 | 1·48±0·10 | 0·435±0·045 | 0·588±0·066 |
| Arachis oil, control | 12 | 1·34±0·09 | 1·32±0·09 | 0·411±0·036 | 0·420±0·075 |
| BHT | | 0·92±0·02* | 0·98±0·09 | 0·363±0·012 | 0·510±0·051 |
| BHA | | 1·16±0·06 | 1·07±0·08 | 0·420±0·024 | 0·492±0·063 |
| Arachis oil, control | 16 | 1·50±0·07 | 1·28±0·02 | 0·309±0·013 | 0·480±0·126 |
| BHT | | 1·39±0·09 | 1·38±0·05 | 0·375±0·051 | 0·393±0·036 |
| BHA | | 1·43±0·05 | 1·25±0·06 | 0·309±0·018 | 0·399±0·125 |

Details of rats and treatment are given in legends to Figs. 2 and 3. Each result is the mean ±SE for 6 animals, in units representing the decomposition of 1 μmole of substrate/hr/mg protein. Figures marked with asterisks differ significantly from those of controls: $^{*}P<0{\cdot}05$; $^{**}P<0{\cdot}01$.

In regard to histology, the slight degrees of fatty change observed in the livers of animals treated with BHA or BHT (Table 3) are not considered significant. In contrast to the changes in enzyme activity observed by biochemical methods, the histochemical assessments

Table 3. *Histochemical and other changes in livers of rats fed BHA or BHT at 0·1% of the diet for 16 wk*

| Compound | Duration of feeding (wk) | Fatty change | | Glucose 6-phosphatase | | Glucose 6-phosphate dehydrogenase | | Acid phosphatase | |
|---|---|---|---|---|---|---|---|---|---|
| | | M | F | M | F | M | F | M | F |
| BHT | 4 | I (6) | N | D (3) | N | I (3) | N | N | D (2) |
| BHA | | I (2) | N | D (3) | N | I (1) | N | N | D(3) |
| BHT | 6 | I (1) | I (2) | I (5) | D (2) | N | I (1) | N | N |
| BHA | | I (1) | N | I (2) | D (2) | N | I (1) | N | N |
| BHT | 16 | N | I (2) | N | I (5) | N | I (1) | N | N |
| BHA | | N | N | N | N | N | N | N | N |

Figures in brackets indicate number of animals affected in the groups of males (M) and females (F) each of which comprised 6 animals. Change indicated by N = No change; D = Decreased; I = Increased.

showed a perilobular decrease in glucose 6-phosphatase only in males at 4 wk and only in 50% of these animals. In groups killed at 6 wk some males showed an increase in activity, and some females a decrease, in comparison with control animals killed at the same time. Other enzymes studied showed no significant change.

## DISCUSSION

*Ascorbic acid excretion as an index of processing enzyme stimulation*

Compounds that enhance the activity of processing enzymes also stimulate liver microsomal NADPH oxidase and accelerate the conversion of D-glucose and D-galactose to D-glucuronic acid, L-gulonic acid and L-ascorbic acid (Horowitz & King, 1953; Berg, Mosbach & Schulenberg, 1954; Burns & Evans, 1956; Burns, Conney, Dayton, Evans, Martin & Taller, 1960). In some instances at least, compounds that stimulate ascorbic acid biosynthesis also inhibit its breakdown in the liver (Koch & Klinger, 1963). The outcome of one or other or both of these effects is an increased level of ascorbic acid in the liver, as observed with carcinogenic hydrocarbons by Kennaway, Kennaway & Warren (1944), and a rise in ascorbic acid excretion in the urine. Longenecker, Fricke & King (1940) were the first to show that a large number of unrelated compounds, mainly narcotics, are capable of causing an immediate increase in ascorbic acid excretion lasting 1–2 days.

The duration of the stimulatory action exercised by a substance on ascorbic acid excretion, like the effect on processing enzymes, is probably governed by the degree of persistence of the compound in the body (Dayton, Vrindten & Perel, 1964). The results described here suggest a parallelism between the degree and duration of enhancement of ascorbic acid excretion and the onset of, and course taken by, liver enlargement when stimulation of processing enzyme activity is involved. It is clear that a material such as BHA, that is cleared rapidly from the body, exerts only a transient stimulatory effect on ascorbic acid excretion; while BHT, whose elimination is more gradual, brings about a more prolonged increase in the output of ascorbic acid. In the course of a short-term feeding study, however, involving the administration of BHA and BHT in the diet at a relatively high level (1000 ppm or about 50 mg/kg/day) the effect of BHT dissipated by 16 wk. With BHA there was an effect in females only, and it was no longer manifest after 4 wk.

Comparing these findings with the changes in relative liver weight, a satisfactory degree of correlation is revealed as far as BHT is concerned, considering that the intake of this material was probably in the region of the threshold dose needed to stimulate processing enzyme activity (Gilbert & Golberg, 1965). With BHA, too, there was concordance between the two parameters except in females at 12 wk.

However limited their scope, these experiments have yielded results that are sufficiently encouraging to warrant further work on ascorbic acid excretion as a means of distinguishing hyperfunctional liver enlargement (that is, one associated with the stimulation of microsomal processing enzymes) from toxic liver enlargement. Deichmann *et al.* (1955) measured the output of glucuronates in the urine of rabbits given a single dose (3·2 g/kg) or 6 daily doses (0·3 g/kg) of BHT. In each group a transient increase in output was observed but was not correlated with any increases in liver weight. It is probable, however, that ascorbic acid is a more reliable criterion than glucuronates.

*Application in man*

The observation of enhanced metabolism through the glucuronic acid pathway cannot be carried out in man using ascorbic acid excretion (Burns, Conney & Koster, 1963). The

alternative pathway of glucuronic acid degradation does serve as an indicator, a fact first noted by Enklewitz & Lasker (1935) who found an elevated excretion of L-xylulose in pentosuric subjects given aminopyrine or antipyrine. Another compound on the same pathway is D-glucaric acid, studied by Marsh & Reid (1963) and Aarts (1965). In view of the promising results obtained by Aarts (1965), it is intended to apply this approach in our laboratories to the assessment of the actions of BHA and BHT in man.

*Liver and adrenal responses*

The tests of liver response, both biochemical and histochemical, yielded very largely negative results. There is no evidence that, under the conditions and over the period of the experiment, either BHA or BHT exercises a hepatotoxic action or any other adverse effect on the test animals.

Adrenal enlargement was noted in 2 groups on BHA and 2 on BHT, 3 of the 4 groups being females. Johnson & Hewgill (1961) reported an increase in weight of the left adrenal in male rats given BHA or BHT at dietary levels of 0·1–0·5%, calculated on a dry-weight basis; there was possibly some effect also on the adrenal in females at 0·1–0·2%. A similar observation has been made with respect to BHT by Sporn & Schöbesch (1961) who fed the antioxidant to young rats (sex unstated) at a dietary level of 0·2% and found in addition that the ascorbic acid content of the adrenal glands was higher in BHT-treated rats than in controls. (This is certainly true on an absolute basis or on a basis of body weight; based on weight of gland analysed, the results were (in mg/g): controls, 0·51; BHT-treated, 0·56—a very much smaller difference). Sporn & Schöbesch (1961) regard the adrenal changes as indicating a stress imposed by BHT on the organism. While adrenal enlargement does undoubtedly occur in response to BHA or BHT, our findings with regard to adrenal ascorbic acid content in rats treated with BHA indicated an effect in males but not in females. It is unlikely that these changes can be attributed to stress since, as yet, very little is known concerning the possibility of microsomal processing enzyme stimulation in the adrenal. It may be that the difference between 'hyperfunctional enlargement' of the adrenal and 'stress' is a matter of semantics.

*Growth*

The element of stress needs to be taken into account when considering effects on growth. The presence of BHT in the diet, particularly at high levels, renders the food unacceptable to rats, with consequent reduction in the rate of growth. Pair-feeding of control rats is not a wholly satisfactory solution to this problem if, in addition to reducing dietary intake, the presence of BHT also imposes a stress on the test animals to which the controls are not subject.

In rats given 0·4% BHT in the diet for the first 6 months and 0·8% BHT for the next 6 months, Karplyuk (1960) observed increased growth in males and decreased growth in females. Sporn & Schöbesch (1961) also noted substantial stimulation of growth in rats (sex unstated) given 0·2% BHT in the diet. At very high doses (600 mg/kg BHA; 500 mg/kg BHT, later reduced to 250 mg/kg) for 68–82 days in rats (sex unstated), Karplyuk (1959) found increased growth. In 1962, however, Karplyuk observed weight loss, from the twenty-second day on, with 500–600 mg/kg/day of BHT. Most other workers have reported some retardation of growth at levels of 0·1–0·5% (dry-weight basis) BHA or BHT in the diet (Brown *et al.* 1959; Johnson & Hewgill, 1961) but most of these studies involved the use of 10% coconut oil, 10% lard, 10% heated lard or 20% lard in the diet.

In our own experiments no significant effect on weight was observed with 0·1% of BH[illegible] BHT in the diet. At this level, therefore, whatever the stress engendered by the presence of these compounds, growth was unaffected.

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# Liver Response Tests. V. Effect of Dietary Restriction on a Short-term Feeding Study with Butylated Hydroxytoluene (BHT)*

I. F. GAUNT, D. GILBERT and D. MARTIN

*British Industrial Biological Research Association, Woodmansterne Road, Carshalton, Surrey, England*

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Abstract—A study involving dietary restriction has been made of the effects of butylated hydroxytoluene (BHT; 3,5-di-*tert*-butyl-4-hydroxytoluene) on growth, food consumption, urinary and adrenal levels of ascorbic acid, activity of a number of liver processing and other enzymes, and concentrations of liver and serum lipids. These aspects were compared in rats fed *ad lib.* and in groups receiving 50% of the previous day's food intake of corresponding control animals. BHT was administered daily, by stomach tube at 500 mg/kg for 14 days, followed by a recovery period of 14 days.

BHT-treated rats on restricted or full diet grew as well as their corresponding controls throughout the treatment and recovery phases. Parallel increases were seen in relative liver weight, activities of aminopyrine demethylase, nitroanisole demethylase and hexobarbitone oxidase and in urinary output of ascorbic acid. Food deprivation hardly affected these changes and in general, full restoration to the original state had occurred after 2 wk of recovery. The depression in glucose 6-phosphatase activity (without change in glucose 6-phosphate dehydrogenase) found after BHT treatment was similarly reversed by the end of the recovery period. The weight and ascorbic acid content of the adrenals remained unaffected by the BHT treatment even when food deprivation was superimposed.

The levels of phospholipid, free fatty acid and cholesterol in the liver remained unaffected in rats given BHT with or without food deprivation. There were striking increases in the serum levels of cholesterol and phospholipid in the BHT-treated groups; these increases were no longer manifest after recovery.

## INTRODUCTION

Gilbert & Golberg (1965), observed that butylated hydroxytoluene (BHT; 3,5-di-*tert*-butyl-4-hydroxytoluene) is capable of increasing the relative weight of the liver while at the same time stimulating the activity of microsomal processing enzymes. This effect is obtained under conditions which, according to Sporn & Schöbesch (1961) involve a state of stress to the animal as judged by their finding of an increase in adrenal size and ascorbic acid content. Moreover, as reported by Brown, Johnson & O'Halloran (1959), there is a failure to gain weight normally, the reason for which has not been investigated by these or other workers.

It was considered of interest to study the relationship, over a short period of intensive administration of BHT, between depressed body weight gain and liver weight on the hypothesis that the depressed growth was an artifact produced by using as controls animals that were pair-fed but not stressed in the same way as those given BHT. One possible approach to the problem is that of Buchman (1962), who measured the time of survival of rats on severely restricted diets containing various fats together with 0·02% propyl gallate and drew the conclusion that the biological value of each fat was greatly reduced by the presence of propyl gallate.

*The preceding paper in this series was published in *Fd Cosmet. Toxicol.* 1965, 3, 433.

It was considered advisable to use a less drastic procedure in our experiments, involving dietary restriction to about one-half the intake of animals fed *ad lib.* and to apply the tests outlined in the preceding papers of this series.

## EXPERIMENTAL

*Materials.* Samples of BHA and BHT were obtained from the same sources and conformed to the same specifications as described by Gilbert & Golberg (1965).

*Animals and diets.* The animals used were female SPF weanling rats (body weight 65–85 g) obtained from the Shell Laboratories, Sittingbourne, Kent. They were housed in pairs in plastic metabolism cages and fed the same diet in powder form as detailed previously (Gilbert & Golberg, 1965). Control and BHT-treated rats on restricted diet were given an amount of food equal in weight to one-half of that eaten during the previous 24 hr by the controls on full diet.

*Dosage.* Rats were divided into two control and two test groups as shown in Fig. 1 in which the dosing schedules are also set out. BHT (500 mg/kg) was administered daily by stomach tube as a 10% (w/v) solution in arachis oil at a dose volume of 5 ml/kg. The groups will be designated in the text by the symbols shown in Fig. 1.

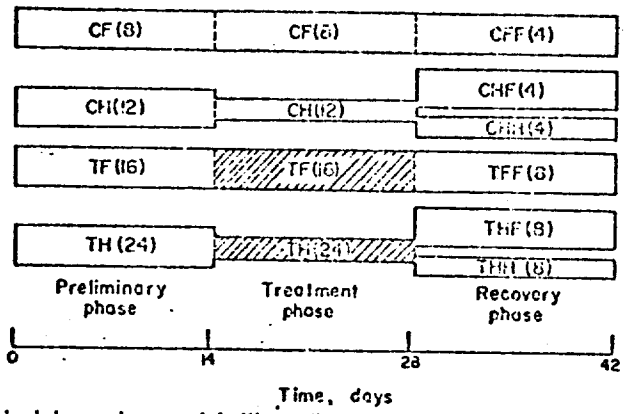


FIG. 1. Treatment schedules and group labelling. First letters C and T indicate control and BHT-treated respectively. Second letters F and H denote full and restricted diet during treatment phase. Third letters F and H signify full and restricted diet during recovery phase. Narrow bands indicate period when animals were on restricted diet, shading indicates treatment (500 mg BHT/kg) and the number of animals in each group is shown in parentheses.

*Conduct of experiment.* Before the experiment commenced, rats were acclimatized for 7 days and during a further period of 7 days, data were obtained on urinary ascorbic acid excretion and growth rate. Growth and output of urinary ascorbic acid were measured daily from day 7 until day 42 which constituted the end of the recovery phase. Food consumption was measured during the treatment and recovery phases.

*Ascorbic acid determinations in urine and adrenals.* The ascorbic acid content of 24-hr urine samples and of adrenals was determined according to the method of Howard & Constable (1965) as modified by Gaunt, Feuer, Fairweather & Gilbert (1965).

*Assay of liver enzymes.* Glucose 6-phosphatase and glucose 6-phosphate dehydrogenase activities were estimated in liver homogenates as described by Feuer, Golberg & Le Pelley (1965). The activities of liver aminopyrine demethylase, nitroanisole demethylase and hexobarbitone oxidase were estimated according to Gilbert & Golberg (1965).

*Estimation of serum lipids.* Blood was taken from the inferior vena cava for serum estimations. Cholesterol was estimated by the method of Zlatkis, Zak & Boyle (1953) as modified by Henley (1957). Serum proteins were precipitated and total esterified fatty acids of the supernatant were estimated by the method of Stern & Shapiro (1953) and the lipid phosphate of evaporated portions of supernatant were determined by the method of Berenblum & Chain (1932) as modified by Martin & Doty (1949).

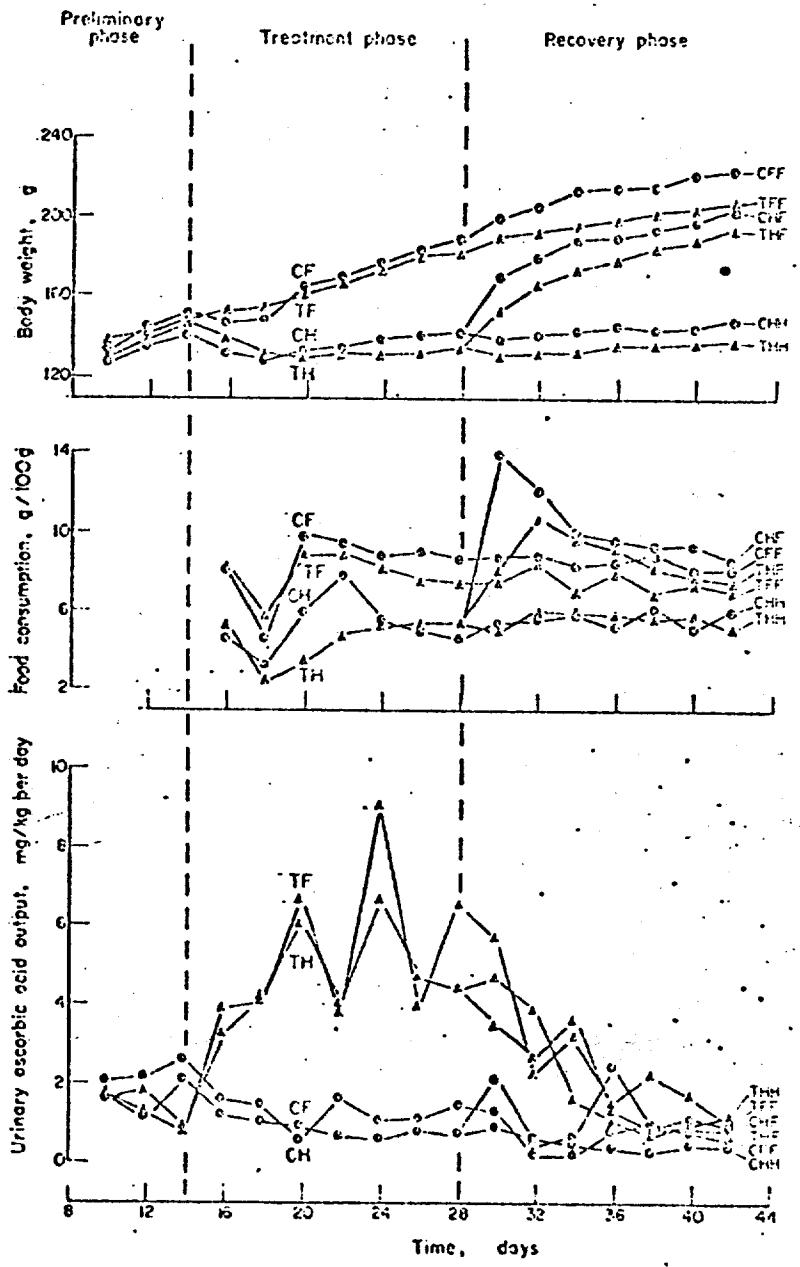


FIG. 2. Body weights, food consumption and daily output of urinary ascorbic acid of control rats (○) or rats treated with 500 mg BHT/kg (▲) and maintained on full or restricted diet, followed by a 14-day period of recovery as shown in Fig. 1. (Values of SE have been deliberately omitted to avoid confusion to the reader.)

*Estimation of liver lipids.* Samples of liver were homogenized in 0·73% (w/v) N (Ansell & Hawthorne, 1964) and the homogenate diluted to 5 ml/g liver. Total cholest of the homogenate and esterified fatty acids and lipid phosphate of homogenate extracts determined by the same methods as described above for serum lipids.

## RESULTS

Despite the massive doses of BHT administered to the treated groups, there was slight if any growth retardation (Fig. 2). The difference from control groups became clearly manifest during the recovery phase, without reaching a level of significance ($P>0$ Considering the fact that BHT-treated rats on restricted diet (group TH) consumed food than the corresponding control group (CH) during the first week of treatment, again on being restored to full diet (THF as compared with CHF), it must be conclude that BHT had no adverse effect on growth.

The effect of BHT on ascorbic acid excretion was striking, beginning almost at once continuing at an enhanced level in all treated groups for about 1 wk after the cessation treatment (Fig. 2). Food restriction had little effect.

### *Liver and adrenal weights*

Liver enlargement occurred as expected in both TF and TH groups in response to administration of BHT (Fig. 3). On an absolute basis, liver weight was, of course, great reduced in groups TH and CH given a restricted diet during the treatment phase; but in relation to body weight, liver weight was reduced in CH, yet remained well-nigh unaffected in TH. At the end of the recovery period there were no important differences between the treated and the corresponding control groups. The liver weights in groups CHF and THF were well maintained, whereas in groups CHH and THH there was a diminution in both absolute and relative liver weights.

Dietary changes affected the weights of the adrenals in a remarkably similar manner in treated and control groups (Fig. 3). After treatment with BHT, the absolute and relative weights of the adrenals of the TF and TH groups remained the same as those of the CF and CH groups respectively. Retardation of growth in restricted groups CH and TH evidently did not affect the absolute weights of the adrenals, so that their relative weights were increased. After recovery there was a difference in absolute adrenal weights between fully-fed and restricted groups which was very striking in the diet-restricted groups CHH and THH; but in relation to body weight the differences were no longer apparent.

Adrenal ascorbic acid levels after the treatment period showed very little difference between treated and control groups (Fig. 4). After recovery, the same was true to a large extent.

Summing up, it may be concluded that BHT brought about an increase in relative liver weight that was no longer apparent after 2 wk of recovery. BHT had no effect on the weight or ascorbic acid content of the adrenals.

### *Liver enzymes*

The effect of BHT on the three liver processing enzymes hexobarbitone oxidase, nitroanisole demethylase and aminopyrine demethylase was a clear stimulation of activity, unaffected or even enhanced by dietary restriction (Fig. 4). The period of recovery served to

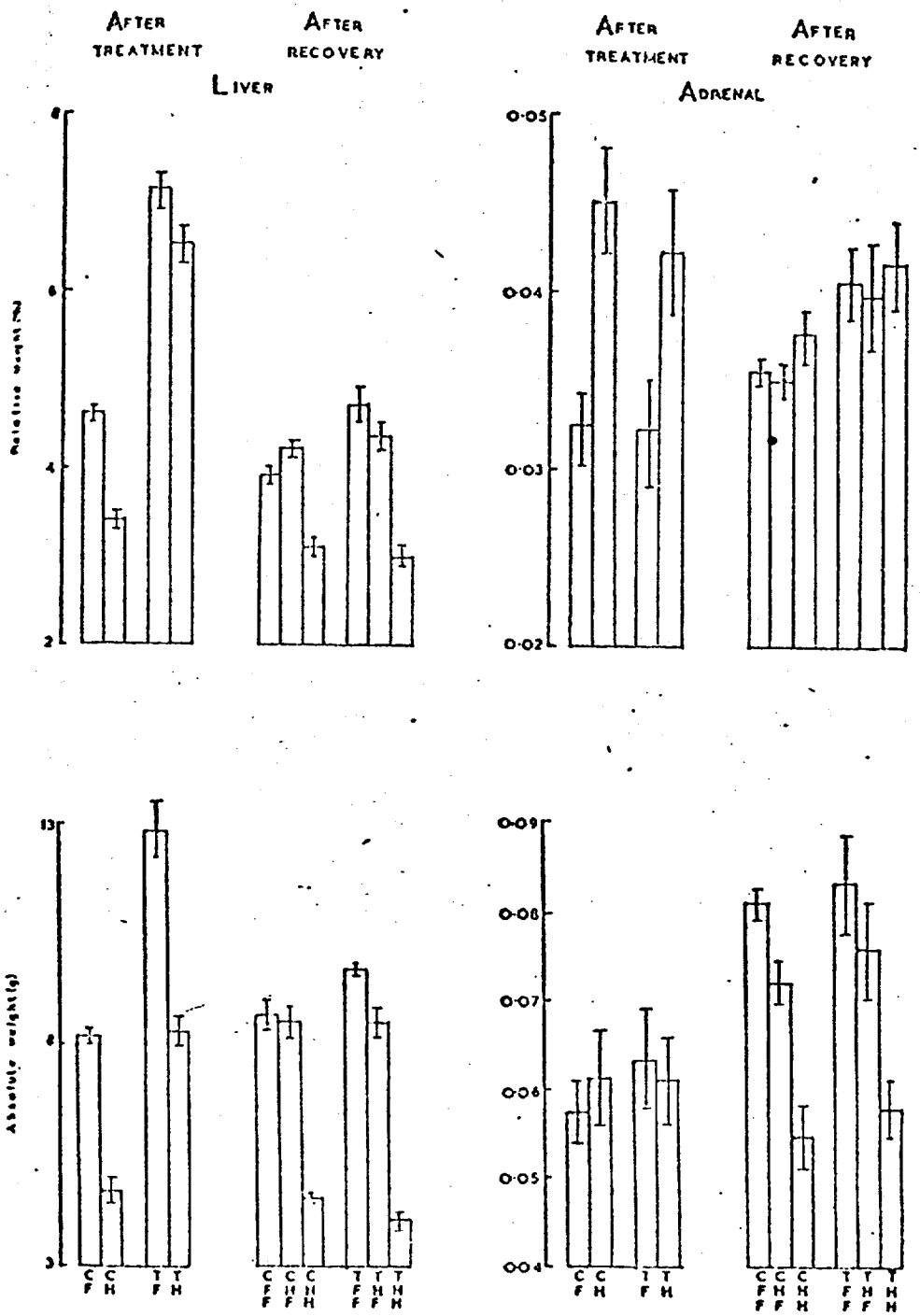


Fig. 3. Relative and absolute weights of liver and adrenals of control and BHT-treated rats receiving the various treatments as shown in Figs. 1 and 2. Vertical bars represent ±SE.

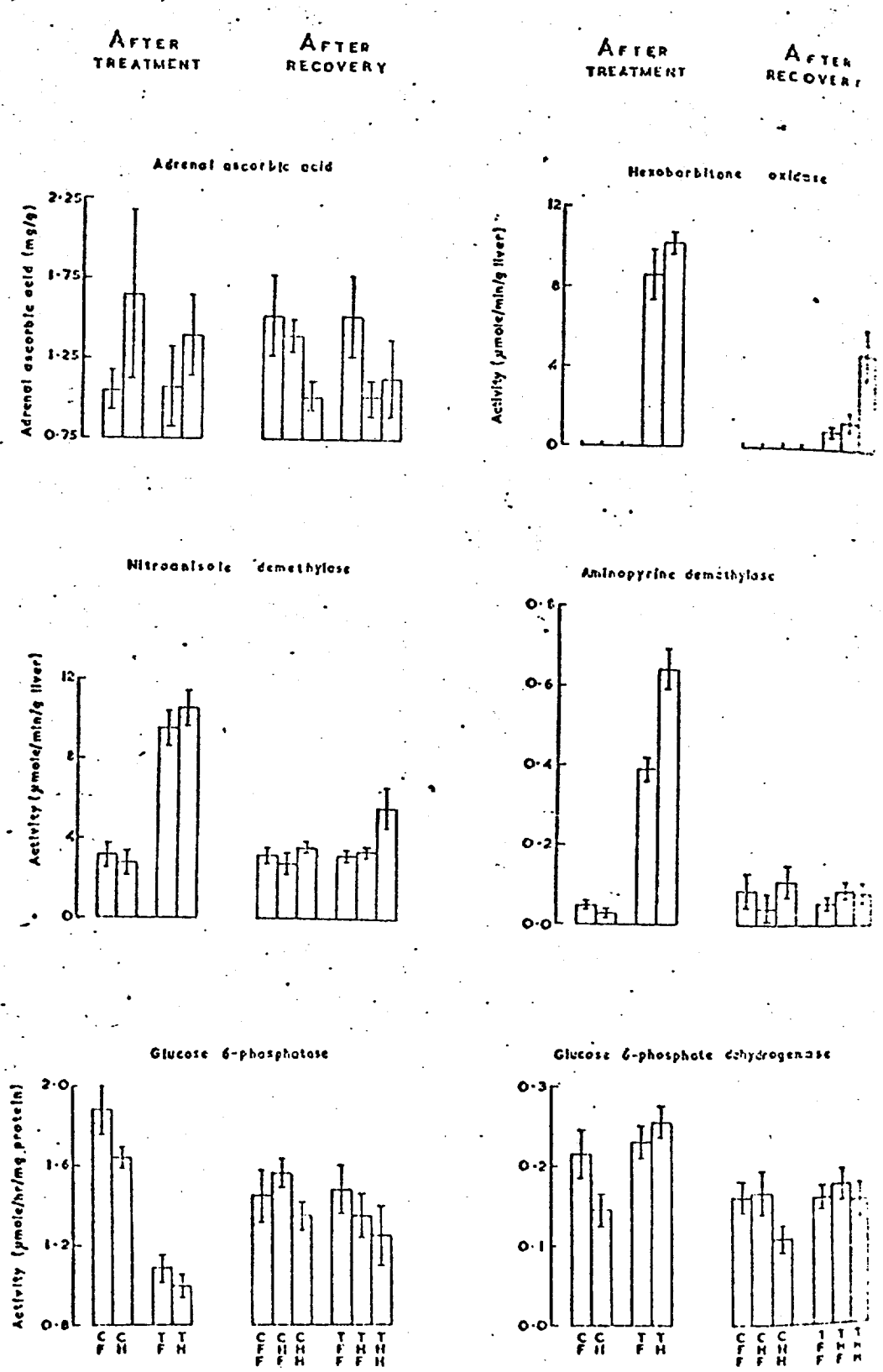


FIG. 4. Adrenal ascorbic acid content and activities of processing and other liver enzymes of control and BHT-treated rats receiving the various treatments shown in Figs. 1 and 2. The enzymes studied were hexobarbitone oxidase, nitroanisole demethylase and aminopyrine demethylase, glucose 6-phosphatase and glucose 6 phosphate dehydrogenase. Vertical bars represent ±SE.

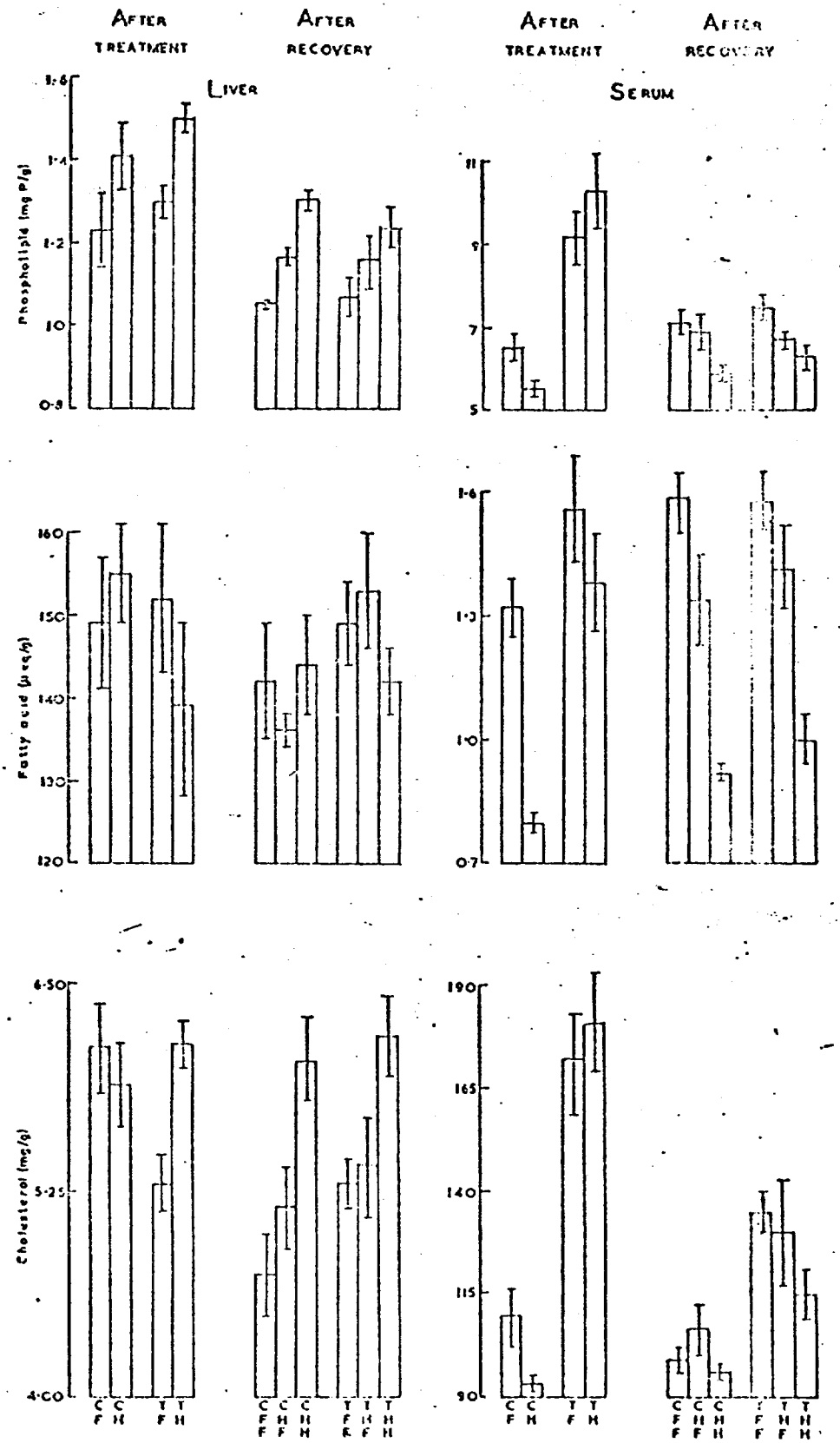


FIG. 5. Phospholipid, fatty acid and cholesterol levels of liver and serum in control and BHT-treated rats receiving the various treatments shown in Figs. 1 and 2. Vertical bars represent ±SE.

eliminate the BHT stimulation except in the THH group where the levels of 2 out of the 3 enzymes remained elevated.

Liver glucose 6-phosphatase was severely depressed after the treatment period but had returned to control levels after the recovery period. There was a tendency for the activities of glucose 6-phosphatase and glucose 6-phosphate dehydrogenase to fall slightly in control groups on restricted diet but the change was not significant. Otherwise the activity of the dehydrogenase enzyme was unaffected by any of the treatments (Fig. 4).

*Liver and serum lipid levels*

The levels of phospholipid, free fatty acid and cholesterol in liver showed remarkably little change as a result of treatment with BHT and/or dietary restriction (Fig. 5). In contrast, the BHT-treated groups TF and TH had greatly elevated serum levels of cholesterol and phospholipid. Dietary restriction, while lowering the serum lipid levels, particularly of free fatty acids, in control group CH, did not affect the serum levels in group TH.

After the period of recovery there remained very little of the rise in serum cholesterol; in fact, the differences between treated and control groups were largely eliminated, leaving only the effect of dietary restriction, especially in groups CHH and THH, to lower serum fatty acids and to a much lesser extent serum phospholipid.

## DISCUSSION

*Starvation as a toxicological tool*

Food restriction has been applied previously in the course of studies devoted to the assessment of safety-in-use of antioxidants. In addition to the work of Buchman (1962) noted above, Sporn & Schöbesch (1961) subjected BHT-treated adult rats to "hunger" and found that they maintained their weight on a par with that of untreated controls that were also starved; but that the nitrogen content of the liver was reduced in BHT-treated rats. Karplyuk (1962) aggravated the experimental conditions of rats given BHT by depriving them of milk and meat for periods of 14 days during the seventh and eleventh months of the test. He found no effect attributable to BHT treatment.

In the experiment described here, the effect of large doses of BHT was a slight one as far as growth was concerned. Food consumption data suggest that initially the administration of BHT produced considerable anorexia, so that even the restricted amount of food provided for the animals was not consumed; during the early part of the recovery phase the same effect was observed. Day, Johnson, O'Halloran & Schwartz (1959) have commented on the reduced consumption of diets containing BHT, attributed by them to the "hot and peppery nature" of such diets.

Our observation of diminished absolute and relative liver weight in control rats on restricted diet is in keeping with similar findings by Barrows & Roeder (1963), Robbins, Small & Anderson (1965) and Peters & Boyd (1965).

*Dietary restriction and liver enzymes*

The effect of starvation on liver microsomal processing enzymes was studied in mice by Dixon, Shultice & Fouts (1960). In contrast to their observation of depressed enzyme activity, which they associated with loss of enzyme protein in the microsome, the present experiment did not reveal a distinct effect of this sort in control rats and even the opposite effect in BHT-treated rats on diet restriction. Oxidative processing enzymes were measured,

but whereas Dixon *et al.* (1960) starved mice for 36 hr the present work involved a less acute but more prolonged food deprivation in rats.

Conflicting results have been obtained in numerous studies investigating the relationship between acute food deprivation, usually for 13–24 hr, and liver glucose 6-phosphatase activity. Authors working with rats or mice have found activity to be increased (Weber & Cantero, 1954, 1957a, 1957b; Ashmore, Hastings & Nesbett, 1954; Langdon & Weakley, 1955; Freedland & Harper, 1957; Bot, Andrássy, Poresalmy & Vereb, 1965; Windman, Artman & Bekierkunst, 1965). Six days' starvation of rats raised the level of glucose 6-phosphatase in the liver to 25% above that of controls (Weber, Singhal, Stamm & Srivastava, 1965). Measurements of other liver enzymes in rats on reduced dietary intake were carried out by Barrows & Roeder (1963). They found that the activity of (inorganic) pyrophosphatase, now known to be identical with glucose 6-phosphatase (Feuer, Golberg & Gibson, 1965) was unaffected by food restriction. Similarly, in the present experiments, reduction of food consumption had a minimal effect on the levels of glucose 6-phosphatase and glucose 6-phosphate dehydrogenase. The striking reduction in the activity of glucose 6-phosphatase, in both TF and TH groups, that was brought about by the large dose of BHT administered requires further investigation. This aspect will be dealt with more fully in the next communication in this series (Feuer, Gaunt, Golberg & Fairweather, 1965).

*Other effects of BHT*

Previous work (Brown *et al.* 1959, Johnson & Hewgill 1961) had shown that the presence of BHT in the diet of rats reduced their weight gain and food consumption. Conversely, Karplyuk (1959) and Sporn & Schöbesch (1961) found increased weight gain and food consumption. In previous work from our laboratory, Gaunt *et al.* (1965) found no difference in these parameters when BHT was fed at a dietary level of 0·1% for 16 wk.

Brown *et al.* (1959) considered that the weight loss found by them with BHT was a toxic manifestation as it was observed even after paired feeding. In the present study, the test material was administered by stomach tube in an attempt to overcome the problem presented by the unpalatability of BHT, whilst the levels of reduction of food intake were controlled. In groups allowed unlimited food, or when food intake was reduced, weight gain in comparison with that of corresponding control groups was affected only to a slight and insignificant degree.

Relative liver weight was increased after BHT treatment and the effect was accentuated in the diet restricted group. In group TFF the return to control level was not quite complete after the recovery period but the other groups were fully restored. In parallel with the effects on relative liver weight, BHT also increased the activities of microsomal processing enzymes and the urinary output of ascorbic acid, thus establishing again the close connexion that exists between these effects (Gilbert & Golbert, 1965; Gaunt *et al.* 1965). Food restriction had little influence on these changes, most of which were completely reversed by the end of the recovery period. It is tempting to suggest that the persistence of slightly elevated processing enzyme activity in the THH group may have been due to mobilization of BHT stored in body fat (Gilbert & Golberg, 1965) as a result of continued dietary deprivation during the recovery period.

Contrary to the evidence suggested by Sporn & Schöbesch (1961) the weight and ascorbic acid content of the adrenals were not affected by the administration of BHT, even when food restriction was superimposed. Under the conditions employed in the present experiment, no

detectable state of stress to the animals resulted from the administration of very large of BHT.

*Effect of BHT on liver and serum lipid levels*

A report of increased serum cholesterol and phospholipid levels was published by D *al.* (1959) who fed male rats on diets, with or without lard supplements, containing BHT (dry weight basis) for 5 wk. In a longer study extending over more than 6 m female rats given 0·1% BHT in a diet supplemented with 10% lard were found to serum cholesterol level 9% greater than that of controls on the same diet without BHT. this increase (significant at the 5% level) was probably fortuitous is suggested by absence of any corresponding increase in serum phospholipid and by the fact that a lard supplement under the same conditions produced a fall of 8% (not significant) in cholesterol. Compare this result, for instance, with the shorter study on males using BHT, in which both cholesterol and phospholipid levels were greatly enhanced by the lard supplement and even more so by 20% lard. At the 0·1% level of BHT, the 5-wk revealed a rise in serum cholesterol and phospholipid which was no longer evident with or 20% lard. It is probably justifiable to conclude that in the experiments of Day *et al.* the 0·1% level of BHT had no effect on these serum lipid components.

In the results recorded here, following 14 days of BHT at 500 mg/kg daily, the serum cholesterol and phospholipid was quite distinct in both fully-fed and diet rats. After recovery, a slight elevation of serum cholesterol level persisted. It should pointed out that these changes moved in parallel with the corresponding changes in liver weight, suggesting that the effects might be linked. From work carried out by Kohn, Kay & Calandra (1965), it is clear that the incorporation of BHT in the diet of for 28 wk at levels at and below 1000 ppm has no effect on serum cholesterol. Only a increase was evident at the 3000 ppm level.

As well as reducing liver weight (Okey, Scheier & Reed, 1960; Mayfield & Roehm, overnight fasting of rats affects the liver lipids and elevates the serum cholesterol level ratio of phospholipid to cholesterol (Mayfield & Roehm, 1961). The effect of fasting 24 hr or more is to impair the capacity of the liver to incorporate acetate into cholest The less severe conditions of food deprivation employed in the present study, while suffic to reduce absolute and relative liver weight in control rats and to reduce serum fatty had surprisingly little effect on liver lipids or other serum lipids.

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# Liver Weight and Microsomal Processing (Drug Metabolizing) Enzymes in Rats Treated with Butylated Hydroxytoluene or Butylated Hydroxyanisole

By D. GILBERT and L. GOLBERG. (*British Industrial Biological Research Association, Carshalton, Surrey*)

Compounds such as phenobarbitone, which cause an increase in relative liver weight (RLW) without histopathological evidence of liver damage, are known to induce the synthesis of processing enzymes (Conney & Burns, 1962; Fouts, 1962). In rats treated with high doses of the antioxidant butylated hydroxytoluene (BHT; 3,5-di-*tert.*-butyl-4-hydroxytoluene) we have found a close relationship to exist between increased RLW, processing enzyme activities, urinary excretion of ascorbic acid and mobilization of stored BHT from abdominal fat. With butylated hydroxyanisole (BHA; 3-*tert.*-butyl-4-hydroxyanisole) also there was a rise in RLW and urinary ascorbate.

After two daily oral doses of BHT (500mg./kg.) there were significant increases in RLW, aminopyrine, demethylase, hexobarbitone oxidase, nitroanisole demethylase (Gilbert & Golberg, 1965) and urinary ascorbic acid (Harris & Olliver, 1942). The BHT content of abdominal fat, as measured by the method of Daniel & Gage (1965), in females and males (mean ±S.E.) was then 230±31 and 162± [illegible] p.p.m. respectively. Despite continued administration of BHT, over the next two days the level of fat fell to about 100 p.p.m. in both sexes while the RLW, enzyme activities and urinary ascorbate continued to rise. No further changes were observed up to the twenty-first day of treatment.

When female rats were treated with BHT for one week, the threshold dose for increases in RLW, aminopyrine demethylase and hexobarbitone oxidase lay between 25 and 75mg./kg./day. Below this threshold the BHT retention factor [ratio of BHT content of fat to dose of BHT] was 0·75 but at higher doses the retention factor fell, reaching 0·19 at 500mg./kg. These observations suggest that a rise in the rate of BHT metabolism had occurred which paralleled the increase in RLW and enzyme activity.

In rats treated with BHA (500mg./kg.) both RLW and urinary ascorbate were elevated but returned to normal by the sixth day of treatment. Treatment for 12 weeks with BHA at this dose caused liver enlargement but no increase in the activities of processing enzymes.

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# Liver Response Tests. III. Liver Enlargement and Stimulation of Microsomal Processing Enzyme Activity*

D. GILBERT and L. GOLBERG

*British Industrial Biological Research Association, Woodmansterne Road, Carshalton, Surrey, England*

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**Abstract**—In female rats given butylated hydroxytoluene (BHT; 3,5-di-*tert*-butyl-4-hydroxytoluene) by stomach tube (500 mg/kg daily for 84 days) liver enlargement was associated with increased activity of the microsomal processing (drug metabolizing) enzymes aminopyrine demethylase, hexobarbitone oxidase and nitroanisole demethylase; a similar effect was observed in mice. Rats treated with butylated hydroxyanisole (BHA; 3-*tert*-butyl-4-hydroxyanisole) had enlarged livers, but no change in processing enzyme activities was observed.

Investigation of the time course and dose-response relationship of the BHT effect revealed a close correlation between increased relative liver weight (RLW), enhanced processing enzyme activity and mobilization of stored BHT from abdominal fat. In rats of both sexes given BHT as above for up to 21 days, RLW and processing enzyme activities were significantly raised by the second day, by which time BHT content of fat had attained values of 230 ppm in females and 162 ppm in males. On days 3 and 4, RLW and enzyme activities continued to rise but the BHT content of fat fell to about 100 ppm in both sexes, remaining at this level until the end of treatment.

In female rats given BHT, the threshold for increases in RLW and processing enzyme activity lay between 25 and 75 mg/kg daily for 7 days. At this threshold a change took place in the relationship between dose and level in body fat: up to 25 mg/kg daily, the BHT content of fat rose in proportion to the dose, reaching 19 ppm; but, with increasing dosage above the threshold, the rate of increase of BHT in fat was reduced, so that the level was only 97 ppm at a dose of 500 mg/kg daily.

The significance of these findings is discussed in relation to the assessment of safety-in-use. It is concluded that liver enlargement induced by BHT is a physiological response.

## INTRODUCTION

The value of organ weight measurements as efficient criteria of stress in toxicological testing was established in the decade before 1952, when Smyth, Weil, Adams & Hollingsworth published a survey of 15–18 yr experience in their laboratories. The results of these workers, combined with subsequent findings by Rowe, Wolf, Weil & Smyth (1959) and Weil & McCollister (1963) have served to demonstrate that, in short- and long-term tests, liver weight was affected more often than any other single parameter investigated. In many instances hepatomegaly was the only positive outcome of the test. The question of the interpretation of an increase in liver weight, in relation to body weight, thus assumes particular importance, especially when other criteria fail to establish whether or not liver damage has occurred.

At the present time we know of three principal situations in which hepatomegaly may occur: it may be associated with actual or incipient liver damage which can be observed histologically; occasionally it may be caused by the storage of macromolecular material in a

*The first 2 papers in this series were published in *Fd Cosmet. Toxicol.* 1965, 3, 235 and 251.

greatly hypertrophied reticulo-endolethial system, a form of thesaurosis which can be confirmed histologically; or, in many instances, the liver may become enlarged without any observable histopathological abnormality.

Many of the compounds which cause this last effect are themselves metabolized by the liver, and early workers regarded the formation of additional normal liver tissue as a 'work hypertrophy', by analogy with the enlargement of muscles in response to exercise. Detailed studies, reviewed by Brodie (1962), Conney & Burns (1962), Fouts (1962), Remmer (1964) and others have revealed that this so-called 'work hypertrophy' is a well-defined response by the liver to a wide variety of exogenous compounds, taking the form of an increase in liver weight and the production of highly active 'processing enzymes'*. The connexion between this effect and the actual "work" done by the liver turns out to be fortuitous in that the compounds which bring about liver enlargement are all lipid-soluble, a property they have in common with compounds acted on by the processing enzymes. The list of substances causing this type of liver enlargement therefore overlaps the list of processing enzyme substrates to a considerable extent, but there are efficient inducers of liver enlargement which are not metabolized (e.g. barbitone) and substrates of processing enzymes which do not cause liver enlargement (e.g. zoxazolamine; Conney, Davison, Gastel & Burns, 1960). We consider it preferable, therefore, to use the term 'hyperfunctional enlargement' to describe this phenomenon.

Compounds that induce the synthesis of additional processing enzymes bring about a corresponding increase in the rate of metabolism and reduction in tissue concentrations of materials which are metabolized by processing enzymes in the liver (Conney *et al.* 1960; Remmer, 1963).

There is clearly a need to develop tests and other criteria that will permit a distinction to be drawn between liver enlargement due to hepatotoxicity and enlargement associated with processing enzyme stimulation. Previous communications from this laboratory have reported certain enzymic changes associated with incipient liver damage caused by the treatment of rats with known hepatotoxins such as coumarin (Feuer, Golberg & Le Pelley, 1965a,b) which does not bring about an increase in processing enzyme activity (Feuer, Gaunt, Golberg & Fairweather, 1965). In this and the following papers in the series (Gaunt, Feuer, Fairweather & Gilbert, 1965; Gaunt, Gilbert & Martin, 1965; Feuer *et al.* 1965) we report some enzymic changes including increases in the activities of certain processing enzymes, observed in rats treated with 2 food additives [butylated hydroxyanisole (BHA; 3-*tert*-butyl-4-hydroxyanisole) and butylated hydroxytoluene (BHT; 3,5-di-*tert*-butyl-4-hydroxytoluene)] that are known to cause liver enlargement without histopathological evidence of hepatic damage (Deichmann, Clemmer, Rakoczy & Bianchine, 1955; Brown, Johnson & O'Halloran, 1959).

In the present study we present evidence that, in rats treated with BHT, there are close correlations between liver enlargement, increases in the activities of processing enzymes and changes in the level of stored antioxidant in body fat.

Although the liver enlargement caused by BHA and BHT has been interpreted as a toxic manifestation by some authorities considering the safety-in-use of these compounds, our

*Other authors have used the term "drug metabolizing enzymes" to refer collectively to this group of related enzymes acting on compounds of exogenous origin and located in the endoplasmic reticulum of liver parenchymal cells. Since these enzymes are capable of bringing about the transformation of a wide variety of compounds other than drugs, it is proposed to refer to them as "processing enzymes" to distinguish them from other, apparently functionally and spatially unrelated microsomal enzymes such as glucose 6-phosphatase, esterase and NADP cytochrome reductase.

findings have made it appear likely that the liver enlargement caused by BHT may be the expression of a physiological response rather than a toxic manifestation.

BHA also caused liver enlargement, but no increase in the activities of processing enzymes was detected. The significance of this discrepancy will be discussed.

## EXPERIMENTAL

*Materials.* BHT ("Topanol" grade) was obtained from Imperial Chemical Industries Ltd., Alderley Park, Cheshire, and from May & Baker Ltd., Dagenham, Essex who also provided BHA (BP grade). The sample of BHT was found to be 99·4% pure and ran as a single spot ($R_F$=0·85) on a thin-layer (silica gel) chromatogram developed in petroleum spirit (40–60°) and sprayed with Gibb's reagent and antimony pentachloride (C. G. Hunter, personal communication in 1965).

Analytical grade reagents were used with the following exceptions. Laboratory reagent or pharmaceutical grades of the following materials were used without further purification: arachis oil, NADP, glucose 6-phosphate, codeine, hexobarbitone, sodium silicate and *o*-cresol. Ether, heptane, isoamyl alcohol and ethyl acetate were washed with 1N-NaOH, 1N-HCl and 3 changes of water; *o*-nitroanisole was washed as above, dried over anhydrous $Na_2SO_4$ and fractionated under reduced pressure; aniline was fractionated under reduced pressure; aminopyrine was recrystallized from petroleum spirit; nicotinamide was recrystallized from benzene-acetone (3:2, by vol.).

*Animals and diet.* Male and female rats of the Carworth Farm SPF strain (100–150 g) were given Spiller's Small Laboratory Animal Diet and water *ad lib.* Some preliminary experiments were conducted using some males, but for the most part female rats of the Porton SPF strain (initial body weight 100–150 g) and female albino mice of the Tuck strain.

*Dosage.* BHA and BHT were administered daily, by oral intubation, at the dose levels stated in the Results section. The test substances were dissolved in arachis oil so that the stated dose was contained in 5 ml/kg body weight of the vehicle. Control animals received orally 5 ml/kg/day of arachis oil.

*Preparation of liver homogenates.* Rats were killed by asphyxiation under nitrogen and the liver exsanguinated, removed and homogenized in 3 ml of ice-cold medium (0·25 M-sucrose, 0·1 M-sodium phosphate, 0·001 M-EDTA, pH 7·4) per g liver. The homogenate was filtered through mesh.

*Enzyme preparation and assays.* The methods which have been used by other workers to assay aniline hydroxylase (AH), hexobarbitone oxidase (HO), nitroanisole demethylase (NADM), codeine demethylase (CDM), and aminopyrine demethylase (APDM) either require very long incubation times, during which the initial reaction rate is not maintained (Cooper & Brodie, 1955; Mitoma, Posner, Reitz & Udenfriend, 1956; La Du, Gaudette, Trousof & Brodie, 1955; Axelrod, 1955; Hart & Fouts, 1963) or are not sensitive enough for the activities found in our strain of rat (Netter, 1960). Modified methods were therefore developed which enabled the initial reaction rate to be measured. The methods described below have been checked to ensure that (1) the optimum pH was used, (2) all cofactors and substrates were present in saturating or optimum concentrations, (3) the activity measured was proportional to the amount of homogenate added, at least up to the amount used under the conditions described below. The results thus obtained should therefore relate directly to the amount of enzyme present *in vivo*, and be less sensitive to changes in the stability of the enzyme or its affinity for substrate and coenzymes than those obtained by methods used previously. All assays were conducted at 37°C under an atmosphere of air with shaking at

120 cycles/min. Replicate incubations were stopped after various intervals from the time the substrate was added (up to 30 min for the APDM assay, up to 15 min for the other assays) and the initial rate obtained from the time course of the reaction.

Details of the composition of the incubation mixtures employed in the assay of NADM, APDM, CDM, AH and HO are provided in Table 1.

Table 1. *Composition of incubation mixtures*

| Incubation mixture | Enzyme activity measured | | | | |
|---|---|---|---|---|---|
| | NADM | APDM | CDM | AH | HO |
| Potassium phosphate buffer (83·5 mM) pH | 7·9 | 7·4 | 7·4 | 7·4 | 7·4 |
| $MgCl_2$ (mM) | — | 3·33 | 3·33 | 10 | 3·33 |
| Nicotinamide (mM) | 16·7 | 8·35 | 16·7 | 25 | 16·7 |
| NADP (μM) | 83·5 | 83·5 | 83·5 | 250 | 83·5 |
| Glucose 6-phosphate (mM) | 2·5 | 2·5 | 2·5 | 6·0 | 1·25 |
| Glucose 6-phosphate dehydrogenase (IU/ml) | 0·06 | 0·06 | 0·06 | 0·02 | 0·06 |
| Substrate and concn (mM) | *o*-Nitroanisole 3·33 | Aminopyrine 0·667 | Codeine 3·33 | Aniline 10 | Hexobarbitone 9·6 |
| Homogenate (ml) | 0·25 | 1·0 | 1·0 | 0·8 | 2·0 |
| Final volume (ml) | 3·0 | 3·0 | 3·0 | 2·0 | 3·0 |

For details see text.

NADM: After incubation the mixture was shaken for 10 min with ether (10 ml) and an excess of solid NaCl, and centrifuged. The ether extract (8 ml) was then shaken with 0·5 ml of 0·1N-NaOH for 10 min and centrifuged. The ether was removed by aspiration and if the aqueous phase was cloudy, air was drawn through the tube until the aqueous phase cleared. The optical density was determined at 430 mμ in a Beckman-Spinco microcolorimeter (light path 0·64 mm). 0·1 μmole of *o*-nitrophenol in the original 3-ml sample gave a reading of 0·228 when this procedure was followed.

APDM: After incubation the mixture was shaken thoroughly with 3 ml of 10% (w/v) trichloroacetic acid and centrifuged. 0·5 ml of 0·2% $NaNO_2$ was added to the supernatant (5ml) and after mixing, the tubes were allowed to stand in crushed ice for 10 min. Then 0·5 ml of 1% ammonium sulphamate was added and after mixing, the tubes were allowed to stand for 3 min before 0·1 ml of 5% ethanolic 1-naphthol and 1·0 ml 4N-NaOH were added, the contents mixed and kept for 10 min. 2 ml of HCl/trichloroacetic acid (1 vol. of conc. HCl plus 1 vol. of 50% (w/v) trichloroacetic acid) and 5 ml of toluene were added, the tubes stoppered and shaken for 10 min. The optical density of the toluene phase was measured at 540 mμ in a Unicam SP 600 spectrophotometer. 0·1 μmole of 4-aminoantipyrine in the 3-ml sample gave an optical density of 0·356 with a 1-cm light path when this procedure was followed.

CDM: After incubation the mixture was shaken for 10 min with ethyl acetate (5 ml) and an excess of solid $NaHCO_3$. 0·5 ml of silicomolybdate reagent was added to the organic phase (4 ml). (The silicomolybdate reagent was prepared as described by Snell & Snell (1954) except that silicate equivalent to 7 g silica/l of reagent was used.) After shaking for 10 min, 0·5 ml of 2·5 M-ammonia was added, the tube contents thoroughly mixed and centrifuged. The organic phase was removed by aspiration and the optical density of the aqueous phase was determined at a suitable wavelength. The absorption spectrum had a

very broad peak with max absorption at 700 mμ, but good results were obtained in the Beckman-Spinco microcolorimeter at 650 mμ. 0·1 μmole of morphine in the 3-ml sample gave an optical density of 0·11 with a 0·64-cm light path when this procedure was followed.

HO: After incubation the mixture was shaken for 10 min with heptane (10 ml) containing 1·5% isoamyl alcohol, 1 ml of 1M-$NaH_2PO_4$ and excess solid NaCl and centrifuged. The heptane phase (2 ml) was shaken with 6 ml of buffer (200 ml of 0·8 M-$Na_2HPO_4$ and 8·5 ml of 10N-NaOH) at pH 11 for 10 min and centrifuged. The organic phase was removed by aspiration and the optical density of the aqueous phase read at 245 mμ in a Unicam SP 500 spectrophotometer. 1 μmole of hexobarbitone in the 3-ml sample gave an optical density of 0·25 with 1-cm light path when this procedure was followed.

AH: After incubation the mixture was subjected to the same procedure as described for NADM except that 1% (w/v) *o*-cresol in 0·1 N-NaOH was used for the second extraction. The optical density was measured at 610 mμ in a Beckman-Spinco microcolorimeter. 0·01 μmole of 4-aminophenol in the 2-ml sample gave an optical density of 0·127 with a 0·64-cm light path.

*Determination of BHT content of fat.* Abdominal fat was collected from around the kidneys and uterus, ovaries or epididymes. The BHT content of the fat was determined colorimetrically by the method of Daniel & Gage (1965).

*Estimation of liver protein.* The method of Lowry, Rosebrough, Farr & Randall (1951) was used to determine liver protein.

## RESULTS

### *Preliminary studies with BHT*

Some preliminary experiments were carried out in mice and rats to investigate the ability of BHA and BHT to increase the activity of liver processing enzymes. The methods employed were basically the same as those used in the main study reported here, but differed slightly in certain details such as total assay volume, cofactor concentrations, vehicle for the administration of test substances, or in the use of isolated microsomes instead of whole homogenate. Since the results of the full study are in close agreement with these earlier findings, the differences of procedure appear to be of no consequence, and the results obtained in these preliminary studies are presented in outline in Tables 2 and 3 as evidence that the effects reported have been obtained consistently and reproducibly in our laboratory.

It can be seen from Table 2 that BHT caused an increase in relative liver weight (RLW) and in liver processing enzyme activity in rats and mice. The raised RLW became discernible in rats 2 days after treatment with 500 mg BHT/kg/day (Table 3). In the same group of

Table 2. *Relative liver weight and processing enzyme activities in rats and mice treated with BHT*

| Species | Sex | Dose (mg/kg/day) | Duration of treatment (days) | Enzyme activity as percentage of control value | | Relative liver weight as percentage of control value |
|---|---|---|---|---|---|---|
| | | | | NADM | AH | |
| Mouse | Female | 750 | 18 | 198* | 242** | 129* |
| Rat | Male | 125 | 6 | 139** | 129** | 118** |
| Rat | Female | 500 | 6 | 178** | 150** | 114* |

Values marked with asterisks differ significantly from those of controls: * $P<0·05$; ** $P<0·01$. Enzymes were assayed in isolated liver microsomes except for male rats where whole liver homogenate was used.

Table 3. *Changes with time of relative liver weight and BHT content of abdominal fat*

| Duration of treatment (days) | Relative liver weight (%) | | BHT content of fat (ppm) | |
|---|---|---|---|---|
| | Control | BHT | Control | BHT |
| 1 | 5·03(1) | 4·71(2) | 3·3* | 124(2) |
| 2 | 4·87(2) | 5·94(4) | | 265(4) |
| 3 | 4·29(1) | 5·70(1) | 10·0† | 102(1) |
| 5 | 4·94(1) | 6·81(2) | | 103(2) |
| 8 | 4·81(1) | 6·68(2) | 10·6(1) | 117(2) |
| 13 | 5·40(1) | 7·12(1) | 5·9(1) | 50(1) |
| 16 | 4·79(1) | 6·40(1) | 1·0(1) | 130(1) |

Female Porton rats were given BHT (500 mg/kg daily) by stomach tube. Controls received arachis oil. Figures in parentheses indicate no. of animals used.
*Pooled sample from 3 rats.
†Pooled sample from 2 rats.

rats the BHT content of the fat rose to 265 ppm by day 2 and despite continued administration of BHT, fell subsequently to about 100 ppm. These effects were later substantiated and investigated in greater detail in the main study, the results of which are presented in Figs. 1 a,b and 2.

Since AH activity was only slightly enhanced in rats, the enzyme was not studied further (Table 2).

### 84-*Day studies with BHT and BHA*

Use was made of livers from pregnant rats which had undergone prolonged treatment (84 days) with BHA or BHT in the course of teratogenicity studies (Clegg, 1965) and assays were carried out on the processing enzymes NADM, CDM, APDM and HO. Both BHA and BHT brought about an increase in RLW and the protein content of the liver was also raised in BHA-treated rats (Table 4). BHA did not affect the activities of any of the 4 enzymes studied; BHT raised the NADM, APDM and HO activities of the liver but reduced the CDM activity.

### *Time course with BHT*

Following the demonstration in BHT-treated rats of liver enlargement, increased enzyme activity and uptake of the antioxidant into abdominal fat, a detailed study was carried out of the time relationships of these effects in rats given BHT (500 mg/kg/day) for 1, 2, 3, 4, 7, 14 and 21 days. The effects on liver weight and enzyme activity are shown in Figs. 1a and 1b. In both sexes the level of HO was significantly raised after 1 day, and RLW, NADM and APDM were significantly raised after 2 days. The level of BHT in body fat (Fig. 2) reached a peak during 1–2 days, falling thereafter to a considerably lower plateau. In both sexes the mean BHT content of the fat at 3 and 4 days was significantly lower than on the 2 preceding days ($P < 0{\cdot}01$). The mean value at 1 and 2 days was higher in females than in males ($P < 0{\cdot}05$) but thereafter there was no significant difference between the sexes.

By about day 4, RLW and enzyme activities had reached a maximum and thereafter changes in the treated animals paralleled those in the controls, except for the apparently spontaneous rise in APDM in control males from 1 wk on (Figs. 1a and 1b).

Table 4. *Relative liver weight and processing enzyme activities in rats treated with BHA or BHT for 84 days*

| Compound | Relative liver weight (%) | Liver protein (%) | BHT content in fat (ppm) | Enzyme activity (μmole/hr/g liver) | | | |
|---|---|---|---|---|---|---|---|
| | | | | NADM | APDM | HO | CDM |
| Arachis oil, control | 3·65±0·12 | 15·9±0·42 | 2·37±0·47 | 2·73±2·3 | 0·114±0·01 | 1·50±0·43 | 3·62±0·18 |
| BHA | 4·63±0·14*** | 17·9±0·66* | — | 2·98±0·19 | 0·081±0·02 | 0·91±0·17 | 3·36±0·07 |
| BHT | 5·86±0·34*** | 16·1±0·38 | 95·8±7·0 | 5·84±0·64** | 0·357±0·13* | 4·92±0·31*** | 3·05±0·13* |

Pregnant rats were given BHA or BHT (500 mg/kg daily) for 84 days by stomach tube. Results represent the mean ± SE of groups of 4 or 5 animals. Values marked with asterisks differ significantly from those of controls: * $P<0·05$; ** $P<0·01$; *** $P<0·001$.

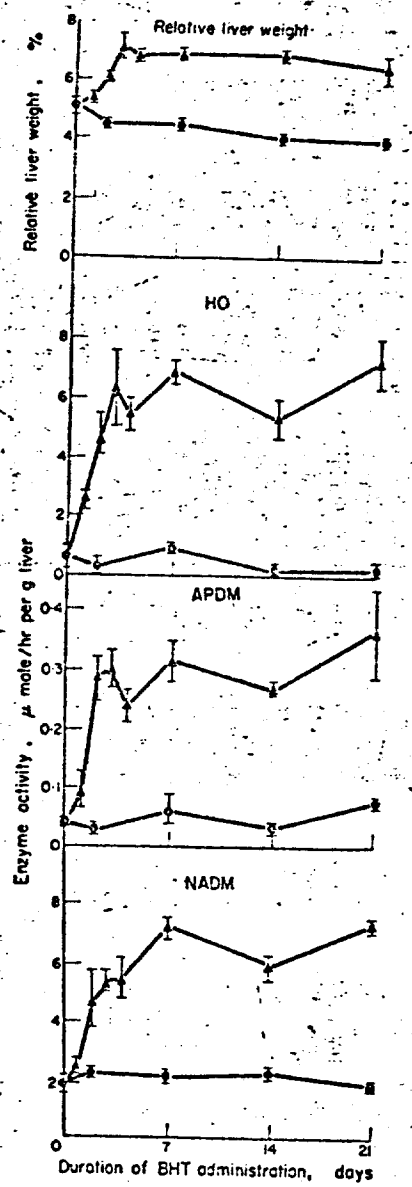


FIG. 1a. Time course of the effect of BHT on relative weight and processing enzyme activities of female rats. Carworth rats were given BHT (▲) at 500 mg/kg or arachis oil (○) daily by stomach tube. Vertical bars indicate SE. Each group comprised 4 rats except at 2 days (6 rats) and 3 days (3 rats).

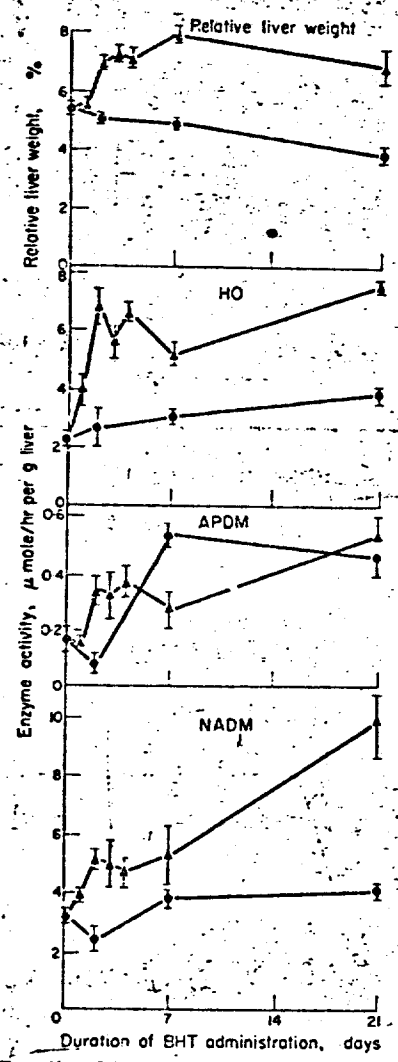


FIG. 1b. Time course of the effect of BHT on relative liver weight and processing enzyme activities of male rats. Carworth rats were given BHT (▲) at 500 mg/kg or arachis oil daily by stomach tube. Vertical bars indicate SE. Each group comprised 4 rats except at 2 days (6 rats), 7 days (6 rats) and 21 days (2 rats).

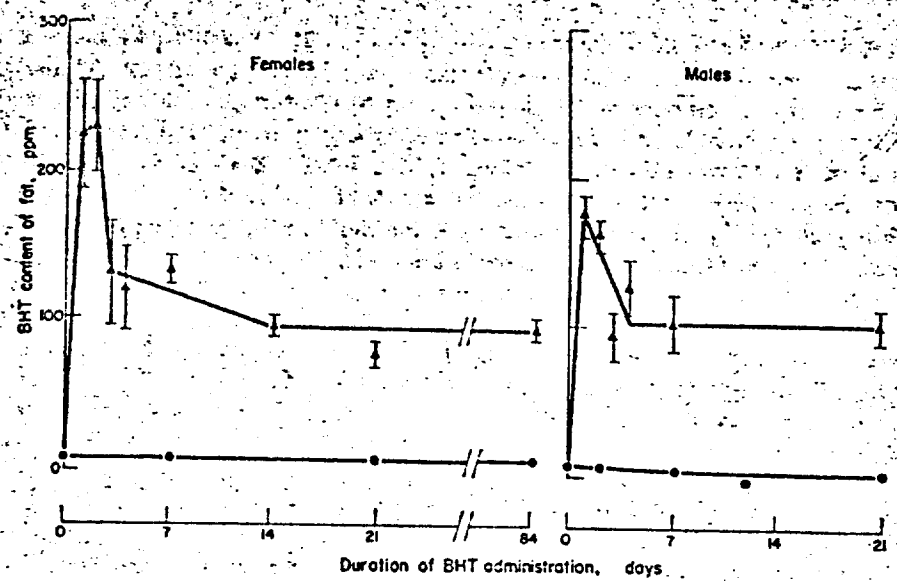


FIG. 2. Changes with time of the BHT content of abdominal fat. For details see legends to Figs. 1a and 1b.

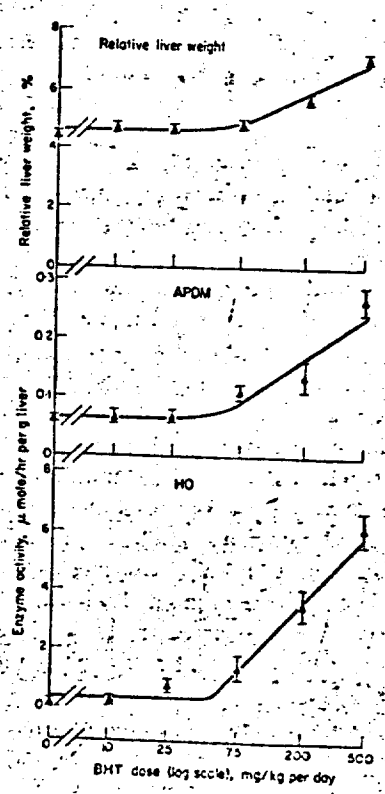


FIG. 3. Effect of various doses of BHT on relative liver weight and processing enzyme activities. Female Carworth rats were given BHT (10–500 mg/kg daily) for 7 days by stomach tube. Vertical bars indicate SE. Each group comprised 4–6 rats. BHT dose is plotted on a logarithmic scale.

*Dose-response curve with BHT*

This experiment was carried out in female rats only, because of the more consistent effect of BHT on enzyme activities noted in this sex and the lower values obtained in female control rats given arachis oil. Groups of 6 females (body weight 100 g) received BHT by oral intubation for 1 wk at dose levels of 10, 25, 75, 200 and 500 mg/kg/day. The threshold dose for liver enlargement and for increased activity of both APDM and HO lay between 25 and 75 mg/kg/day (Fig. 3). At doses above this threshold the percentage of the BHT dose retained in the body fat became progressively lower (Fig. 4).

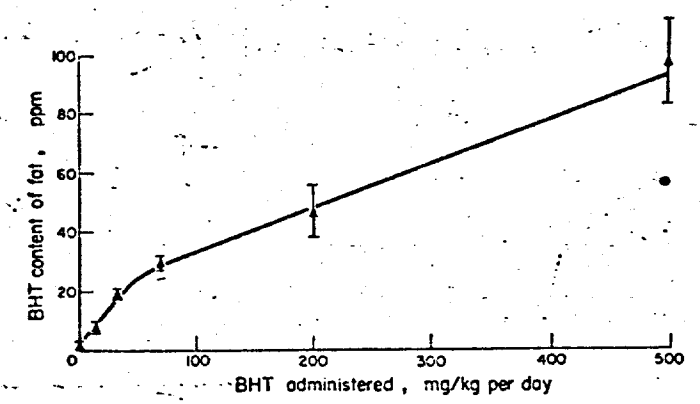


FIG. 4. Effect of various doses of BHT on the BHT content of abdominal fat. BHT dose is plotted on a linear scale. For other details see legend to Fig. 3.

## DISCUSSION

The present observation of enhanced processing enzyme activity induced by BHT is the first time that this change has been shown to be brought about by a direct food additive, although the same effect is known to follow treatment with some insecticides, residues of which may remain on agricultural produce (Hart & Fouts, 1963, 1965; Hart, Shultice & Fouts, 1963; Koransky, Portig, Vohland & Klempau, 1964; Ghazal, Koransky, Portig, Vohland & Klempau, 1964).

Once this enzyme had been demonstrated in our preliminary experiments with male and female rats and female mice (Table 2) the main investigation was undertaken to answer 4 questions: how rapidly are the enzyme activities increased; is a maximum effect attained, and if so when; what is the threshold dose; does the stimulation of processing enzyme activity have any effect on the retention of BHT in the body fat? These 4 questions are clearly answered by the results obtained with female rats (Figs. 1a, 2–4). A significant increase in HO activity was observed after 24 hr and all 3 enzymes as well as the relative liver weight were significantly raised by 48 hr. The maximum effect was reached by about the third day of treatment and no further change occurred up to 3 wk. Following these increases in processing enzyme activities the BHT content of the body fat fell to less than one-half of the level reached in the first 2 days.

With daily doses up to 25 mg/kg, liver weight and enzyme activity remained unchanged (Fig. 3) and the BHT content of the body fat rose sharply in direct proportion to the dose (Fig. 4). When the daily dose was 75 mg/kg or higher the liver was enlarged, its HO and APDM activities were raised and the BHT content of the body fat now rose much more slowly, in relation to the dose administered, than it did at low dose levels.

In male rats the course of liver enlargement, enzyme induction and change in BHT content of body fat generally paralleled, in relation to time, the corresponding effects observed in females; but the differences between control and treated animals were less striking in males than in females. This was partly due to the higher level of processing enzymes in untreated males (Quinn, Axelrod & Brodie, 1958) and partly also to the greater sensitivity of males to high doses of BHT. Some of the animals in the 7-day group were in poor condition when killed, and this seems to have been reflected in low levels of enzyme activity. The sudden rise in APDM in control males at 7 days might have been connected with the onset of puberty (these rats weighed about 180 g when killed). It cannot be ascribed to the administration of arachis oil, because equally high APDM activities were found in untreated males of similar body weight. This sudden rise in enzyme activity resembles that reported by Quinn *et al.* (1958) for HO in males, but not in females. However, only a small rise in HO in males was observed in the present study.These are probably further instances of the sex and strain differences in processing enzyme activity which have been reported previously (Siegert Alsleben, Liebenschultz & Remmer, 1964). The initial peak of BHT storage in body fat in males was not as high as in females (Fig. 3), probably due to the sex difference in processing enzyme activities already referred to.

Turning to the enzymes whose activities were raised after BHT treatment, there have been reports of induction by other compounds of HO (Fujimoto, Blickenstaff & Schueler, 1960; Kato, Chiesera & Vassanelli, 1964), APDM (Conney *et al.* 1960; Hart & Fouts, 1965) and AH (Hart & Fouts, 1965; Kampffmeyer & Kiese, 1963). Although induction of *o*-nitroanisole demethylase has not previously been reported, induction of *p*-nitroanisole demethylase has been brought about by several compounds (Netter & Seidel, 1964) and it is possible that these closely related substrates are metabolized by the same enzyme. CDM, which was not raised in BHT-treated rats, has been reported to be unaffected by the potent inducer of processing enzyme synthesis, 3-methylcholanthrene (Takemori & Mannering, 1958) and no report of induction of this enzyme has appeared in the literature.

Although there were no changes in processing enzyme activities in rats treated with BHA for 84 days a striking parallelism has been observed in short-term experiments with BHA and BHT between the increase in liver weight and the urinary output of ascorbic acid (Gaunt *et al.* 1965). This was just as true in the case of BHA as with BHT and suggests that a stimulation of processing enzyme activity does occur with BHA but that animal to animal variations render it difficult to detect minor and transient changes of this sort.

*Toxicological interpretation*

In order to assess the significance of this effect of BHT on the rat liver it is necessary to consider it in relation to the substantial body of knowledge regarding hyperfunctional liver enlargement which has been built up over the last 10 yr. It is now clear that, depending on the compound used and the conditions of the experiment, stimulation of processing enzymes can take place at varying rates, to different extents, for short or greatly prolonged periods. Invariably, however, the process is reversible when the stimulus is withdrawn. Usually complete reversal occurs within a matter of days of stopping treatment but the return to normal may take several weeks or months when high doses have been given over a prolonged period (Remmer & Siegert, 1964) or the test substance persists within the tissue (Hart & Fouts, 1965). Changes in liver weight appear to follow closely the alteration in processing enzyme activity and are equally reversible. Evidence of the reversibility of the effects of BHT treatment has been obtained in this laboratory (Gaunt *et al.* 1965).

It is legitimate to question whether the type of hyperfunctional liver enlargement under discussion is a manifestation of a physiological response (Conney, Schneidman, Jacobson & Kuntzman, 1965). May it not reflect a form of toxic damage, even when no histopathological evidence can be seen?

More light is thrown on this phenomenon by the evidence forthcoming from electron microscopy, which shows that processing enzyme stimulation by, for example, the administration of phenobarbitone, involves a replacement of rough endoplasmic reticulum by cisternae of smooth reticulum (Herdson, Garvin & Jennings, 1964 a,b). Up to a certain stage this is in all likelihood a physiological adaptive response. It is a moot point whether further developments, occurring in response to prolonged high dosage, and involving the formation of myelin figures (Herdson & Kaltenbach, 1965) can still be regarded as physiological processes, even though these further changes still appear to be reversible. Apparently similar myelin figures have been observed in association with necrosis, or inflammatory or malignant change, effects which are not associated with processing enzyme induction. As Herdson & Kaltenbach (1965) point out, it remains to be seen whether these cytoplasmic inclusions have a positive function within the cell or whether they are the end-product of some degenerative process.

Since carcinogenic aromatic polycyclic hydrocarbons such as 3-methylcholanthrene were the earliest compounds shown to produce stimulation of processing enzymes (Conney, Miller & Miller, 1956), it is important to consider whether hyperfunctional changes of this sort are indicative of incipient carcinogenicity, at least in some instances. The study of 57 aromatic polycyclic compounds by Arcos, Conney & Buu-Hoi (1961) failed to reveal any correlation between carcinogenicity and the ability to bring about a rise in azo dye demethylase activity in rat liver. More recent work by Fouts & Rogers (1965) served to emphasize the distinction between the aromatic polycyclic hydrocarbons and other inducing agents such as barbiturates and organochlorine pesticides. The former act in a highly selective manner, so that the activity of some processing enzymes is strikingly enhanced while most remain unaffected, whereas the latter bring about a generalized induction and cause the majority of processing enzymes to become increased. In particular, HO is sensitive to this generalized induction but is unaffected by treatment with aromatic polycyclic hydrocarbons.Thus, in increasing the activities of a range of processing enzymes including HO, the effect of BHT resembles that of the barbiturates and organochlorine pesticides, as opposed to the more specific and limited effect of the polycyclic aromatic hydrocarbons.

Finally, it has recently become clear that hyperfunctional liver enlargement may be accompanied by changes in steroid hormone metabolism by the liver (Jellinck & Irwin, 1963; Conney & Klutch, 1963; Conney & Schneidman, 1964). As the steroid hydroxylases share the cofactor requirements and intracellular location of the processing enzymes, it seems likely that this change in steroid metabolism may be a frequent concomitant of hyperfunctional hepatomegaly. However, only some minor pathways of steroid metabolism appear to be affected (Conney & Schneidman, 1964) and it may well be that the physiological significance of this change is slight.

*Practical aspects*

The estimate of the acceptable daily intake of BHT for man has been assessed at a maximum level of 0·5 mg/kg body weight (equivalent to about 30 ppm in total diet), conditional upon the availability of expert control (Joint FAO/WHO Expert Committee on Food Additives, 1962). In practice, the provisions of national legislation (e.g. USA, UK) make

possible an upper limit of intake, averaged over the whole diet, of about 4 ppm if it is assumed that full advantage was taken of all legal tolerances. In the US, where the widest range of dietary applications is permitted for BHT, the actual concentration in the whole diet is 2 ppm (Food and Drug Administration, 1964).

The human intake of BHT is thus unlikely to exceed a few mg daily, say 0·2 mg/kg body weight. Even if it is considered desirable to accept only the no-effect level in rats established in the work described above, namely at least 25 mg/kg, it is clear that a substantial margin of safety exists. It is probably more appropriate to disregard hyperfunctional liver enlargement in assessing the acceptability of BHA or BHT for use in food.

## CONCLUSION

The weight of evidence tends to show that, for all practical purposes, at meaningful levels of dosage, processing enzyme stimulation is a physiological adaptive response by the liver cell; and the same must apply to the liver enlargement which accompanies it.

In the specific instance of BHT, we have found a consistent no-effect level of 25 mg/kg/day with respect to liver enlargement and to the 2 enzymes most sensitive to the stimulatory effect.

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## Tests de réaction hépatiques. III. Hypertrophie microsomique hepatique et stimulation de l'activite des enzymes microsomiques

**Résumé**—Sur des femelles de rats auxquelles on donnait de l'hydroxytoluène butylé (BHT; 3,5-di-*tert*-butyl-4-hydroxytoluène) par tubage gastrique, à raison de 500 mg/kg par jour pendant 84 jours l'hypertrophie du foie était accompagnée par une augmentation de l'activité des enzymes microsomiques (métabolisant les médicaments) suivantes: aminopyrine déméthylase, hexobarbitone oxydase, et nitroanisol déméthylase. On observait un effet analogue sur les souris. Les rats traités à l'hydroxyanisol butylé (BHA; 3-*tert*-butyl-4-hydroxyanisol) avaient des foies dilatés, et on n'observait aucune modification dans l'activité des enzymes.

L'étude de la variation de l'effet du BHT au cours du temps, en fonction de la dose a fait apparaître une relation étroite entre l'augmentation du poids relatif du foie (RLW) l'augmentation de l'activité des enzymes le métabolisant et la mobilisation du BHT emmagasiné dans les graisses abdominales. Sur des rats des deux sexes auxquels on avait administré du BHT comme indiqué ci-dessus, pendant des périodes pouvant atteindre 21 jours, le poids relatif du foie et les activités des enzymes le métabolisant avaient nettement augmenté au second jour. A ce stade, la proportion de BHT dans la graisse atteignait 230 ppm chez les femelles et 162 ppm chez les mâles. Pendant les troisième et quatrième jours, le poids relatif du foie et l'activité des enzymes continuaient à croître, mais la proportion de BHT dans la graisse tombait à environ 100 ppm dans les deux sexes, et restait à peu près à ce niveau jusqu'à la fin du traitement.

Chez les femelles de rats traitées au BHT, le traitement minimal provoquant une augmentation du poids relatif du foie et de l'activité des enzymes le métabolisant etait compris entre 25 et 75 mg/kg par jour, pendant 7 jours. A partir de ce traitement minimal la relation entre la dose et la proportion de BHT dans la graisse est modifiée: jusqu'à 25 mg/kg par jour, la proportion de BHT dans la graisse augmente proportionnellement à la dose, et atteint 19 ppm, mais lorsqu'on continue à accroître la dose au-dessus du seuil, la vitesse d'augmentation de la proportion de BHT dans la graisse diminue, si bien que cette proportion est seulement de 97 ppm avec une dose journalière de 500 mg/kg.

La signification de ces résultats pour déterminer la sûreté d'emploi est discutée. On conclut que l'hypertrophie du foie sous l'effet du BHT est une réaction physiologique.

## Untersuchung der Leberreaktion. III. Lebervergrösserung und Anregung der Aktivität der mikrosomalen abbauenden Enzyme

**Zusammenfassung**—Weibliche Ratten erhielten mit der Magensonde 84 Tage lang täglich 500 mg/kg butyliertes Hydroxytoluol (BHT; 3,5-Di-*tert*-butyl-4-hydroxytoluol). Mit einer Lebervergrösserung trat eine erhöhte Aktivität der mikrosomalen arzneimittelabbauenden Enzyme Aminopyrindemethylase, Hexobarbitaloxydase und Nitroanisoldemethylase auf; eine entsprechende Wirkung wurde bei Mäusen festgestellt. Mit butyliertem Hydroxyanisol (BHA; 3-*tert*-butyl-4-hydroxyanisol) behandelte Ratten liessen zwar eine Lebervergrösserung, aber keine Veränderung der Aktivität der metabolisierenden Enzyme erkennen.

Die Beobachtung des zeitlichen Verlaufs und der Abhängigkeit des BHT-Effekts von der Dosis liessen eine enge Beziehung zwischen dem erhöhten relativen Lebergewicht (RLG), einer erhöhten Aktivität der metabolisierenden Enzyme und einer Mobilisierung des ges-

peicherten BHT aus dem Bauchfett erkennen. Bei Ratten beider Geschlechter, die BHT wie oben angegeben bis zu 21 Tage lang erhielten, waren das RLG und die Aktivität der metabolisierenden Enzyme vom zweiten Tage an signifikant erhöht, wobei gleichzeitig auch der BHT-Gehalt des Fetts die Werte von 230 ppm bei den weiblichen und 162 ppm bei den männlichen Tieren erreicht hatte. Am 3. und 4. Tage stiegen das RLG und die Enzymaktivität noch weiter an, aber der BHT-Gehalt des Fetts sank auf etwa 100 ppm bei beiden Geschlechtern und blieb bis zum Ende der Behandlung auf dieser Höhe.

Bei weiblichen Ratten, die BHT erhielten, lag der Schwellenwert für BHT, gemessen an der Vergrösserung des RLG und der Erhöung der Aktivität der metabolisierenden Enzyme, bei einer Dosis zwischen 25 und 75 mg/kg täglich, 7 Tage lang appliziert. Bei dieser Schwellendosis erfolgte eine Änderung der Beziehung zwischen Dosis und dem Gehalt im Körperfett: bis zu 25 mg/kg täglich stieg der BHT-Gehalt des Fetts proportional zur Dosis und erreichte 19 ppm; wird die Dosis über den Schwellenwert erhöht, ist die weitere Zunahme des BHT-Gehalts im Fett relativ zum höheren Angebot vermindert. Der Gehalt bei einer täglichen Dosis von 500 mg/kg betrug nur 97 ppm.

Die Bedeutung dieser Ergebnisse wurde im Hinblick auf die Beurteilung der Sicherheit während des Gebrauchs diskutiert. Es wurde der Schluss gezogen, dass die von BHT herbeigeführte Lebervergrösserung eine physiologische Reaktion ist.

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# BHT Oxidase. A Liver-microsomal Enzyme Induced by the Treatment of Rats with Butylated Hydroxytoluene

D. GILBERT and L. GOLDBERG*

*British Industrial Biological Research Association, Woodmansterne Road, Carshalton, Surrey, England*

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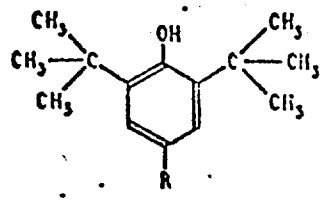
**Abstract**—The ability of an enzyme from rat liver to oxidize butylated hydroxytoluene (2,6-di-*tert*-butyl-4-methylphenol; BHT) has been demonstrated. The reaction product was 2,6-di-*tert*-butyl-4-hydroxymethylphenol (BHT-alcohol). A quantitative assay for this enzyme is described; the enzyme requires NADPH and $O_2$, the pH optimum is 7·4, $Mg^{2+}$ is not required and no activity is obtained with NADH. The enzyme is competitively inhibited by SKF 525A.

Treatment of female rats with BHT (500 mg/kg daily for 5 days) caused a sixfold increase in the activity of BHT oxidase/g liver and a 35% increase in relative liver weight, both prevented by actinomycin D. This increase in BHT-oxidizing capacity accounts for previously-reported changes in tissue retention of BHT.

## INTRODUCTION

Studies of the metabolism of butylated hydroxytoluene (2,6-di-*tert*-butyl-4-methylphenol; BHT) have revealed the nature of the final products of metabolism of this compound in the rabbit and rat. Dacre (1961) found several metabolites in the urine of rabbits treated with BHT including the free 4-carboxyl derivative (BHT-acid) and its glucuronide and glycine conjugates, the glucuronide of the hydroxybutyl derivative and some unidentified ethereal sulphate. Other metabolites have been detected in rabbit urine: the condensation product 4,4′-ethylenebis(2,6-di-*tert*-butylphenol) by Agaki & Aoki (1962) and BHT-aldehyde by Aoki (1962). Wright, Akintonwa, Crowne & Hathway (1965) found that in the rat the same metabolites were formed from BHT as from BHT-alcohol namely, the 4-carboxylic acid and its glucuronide. No hippuric acid derivative was formed. The structures of BHT and its 4-methyl substituted derivatives are given in Fig. 1.




| | |
|---|---|
| BHT | R = $CH_3$ |
| BHT – alcohol | R = $CH_2OH$ |
| BHT – aldehyde | R = CHO |
| BHT – acid | R = COOH |

FIG. 1. Structures of BHT and its 4-methyl substituted analogues.

However, to the best of our knowledge, no studies of BHT metabolism *in vitro* have been reported and the need for such studies became apparent following our observations

*Present address: Institute of Experimental Pathology and Toxicology, The Albany Medical College of Union University, Albany, New York.

of variations in the degree of retention of BHT in abdominal fat. These variations could be interpreted as resulting from adaptive changes in the level of BHT-metabolizing enzymes in BHT-treated rats (Gilbert & Golberg, 1965). When BHT was administered daily to rats, high levels of BHT were observed in fat for one or two days but further treatment led to a fall in BHT content of fat. This fall followed a marked rise in relative liver weight and in the activities of some processing ("drug-metabolizing") enzymes in the liver. It seemed likely that the activity of enzymes metabolizing BHT in the liver might have undergone a similar increase.

Using a method of assay developed for an enzyme in rat liver that oxidizes BHT to BHT-alcohol, we report changes in the level of this enzyme following oral administration of BHT to rats.

## EXPERIMENTAL

*Materials.* BHT ("Topanol" grade) was obtained from Imperial Chemical Industries Limited, Alderley Park, Cheshire. BHT-alcohol, BHT-aldehyde and BHT-acid were gifts of Dr. A. S. Wright, Shell Chemical Limited, Sittingbourne, Kent. Laboratory grades of NADP, glucose 6-phosphate, cyclohexane and Gibbs reagent (2,6-dichloroquinone-4-chloroimine) were used without further purification. Arachis oil was of edible quality, free from known additives. Nicotinamide was recrystallized from benzene–acetone (3:2, by vol.). Heptane, diethyl ether and isoamyl alcohol were washed with 1N-HCl, 1N-NaOH and three changes of water. All other reagents were analytical grade.

*Animals and diet.* Carworth Farm SPF rats of both sexes were given Spillers Small Laboratory Animal Diet and water *ad lib.*

*Dosage.* The required dose of BHT was given by stomach tube in 5 ml of arachis oil per kg body weight. Actinomycin D was administered intraperitoneally in 5 ml of saline per kg body weight. Controls received vehicle only.

*Preparation of liver microsomes.* Rats were killed by asphyxiation under $N_2$ and the liver exsanguinated, removed and homogenized in 3 ml ice-cold medium (0·25 M-sucrose, 0·01 M-sodium phosphate, 0·01 M-nicotinamide, 0·001 M-EDTA, pH 7.4) per g liver in a smooth-faced pestle type homogenizer with 0·02-in. clearance (Aldridge, Emery & Street, 1960). The homogenate was centrifuged for 20 min at 13,000 *g* and the residue washed with a similar volume of medium. The combined supernatants were centrifuged for 1 hr at 38,000 *g*, the residue washed with a further portion of medium and the microsomal pellet finally suspended in the medium to 1 ml/g liver.

*Total liver fractionation.* This was carried out by the procedure of Feuer, Golberg & Le Pelley (1965).

*Protein estimation.* This was carried out by the method of Lowry, Rosebrough, Farr & Randall (1951).

*Enzyme incubation.* The incubation medium contained 83·3 mM-potassium phosphate pH 7·4, 3·3 mM-$MgCl_2$, 16·6 mM-nicotinamide, 0·083 mM-NADP, 2·5 mM-glucose 6-phosphate, 0·06 IU/ml glucose 6-phosphate dehydrogenase, 0·045 mM-BHT, and microsomes from 0·125 g liver/ml. Incubations were carried out at 37° with shaking at 120 strokes/min, and were made under aerobic conditions unless otherwise stated.

*Thin-layer chromatography.* Metabolites were extracted from the incubation medium by either saturating it with NaCl or acidifying with HCl to 0·25 M and then shaking it with washed diethyl ether. The ether extract was dried with anhydrous $Na_2SO_4$ and the ether evaporated at room temperature under $N_2$. The residue was chromatographed on thin-layer

plates of silica or alumina, both media containing $CaSO_4$ binder and being activated at 160° for 30 min. Silica chromatograms were developed with light petroleum (60–80°)–diethylether (3:2, by vol.) according to Wright *et al.* (1965) and alumina chromatograms were developed with cyclohexane-chloroform (1:1, by vol.) according to J. W. Daniel (personal communication in 1966). Chromatograms were sprayed with Gibbs reagent and $Na_2B_4O_7$ (Wright *et al.* 1965) or with molybdophosphoric acid and ammonia (Dacre, 1961). The same metabolites were found in acid and salt extracts.

*Quantitative estimation of BHT-alcohol.* The incubation medium (3 ml) was shaken for 10 min with excess solid NaCl and 10 ml washed heptane containing 1·5% (by vol.) of washed isoamyl alcohol and centrifuged. The organic phase (8 ml) was shaken for 10 min with 0·5 ml of aqueous $Na_2CO_3$ (0·2 g/100 ml) and 0·5 ml of ethanolic Gibbs reagent (5 mg/100 ml) and centrifuged. The organic phase was removed by aspiration and the optical density of the aqueous phase was read in a Beckman-Spinco 151 Spectrocolorimeter at 580 mμ.

*Enzyme assays.* The incubation medium without substrate (2·9 ml) was shaken aerobically at 37° for 5 min to equilibrate before the reaction was started by the addition of BHT dissolved in 2-methoxyethanol (0·1 ml). The reaction was stopped by shaking with NaCl and the heptane–isoamyl alcohol mixture as described above. Replicates were incubated for different times and the initial reaction rate ascertained.

## RESULTS

### *Identification and estimation of BHT-alcohol*

When BHT was incubated with rat-liver microsomes under the standard conditions, a metabolite was formed which gave a blue colour with the Gibbs reagent. When chromatographed on silica and alumina, as described in the Experimental section, the metabolite ran with the same $R_F$ values and gave the same colour reactions as BHT-alcohol. The reference compounds, BHT-aldehyde and BHT-acid, had respectively higher and lower values than the BHT-alcohol on silica gel (Wright *et al.* 1965) while the hydroxybutyl metabolite of BHT runs much more slowly than BHT-alcohol on alumina (J. W. Daniel, personal communication, 1966) and gives a different colour with molybdophosphoric acid (Dacre, 1961). An authentic sample of BHT-alcohol was carried through the procedure described and the absorption spectrum, against a reagent blank, is shown in Fig. 2.

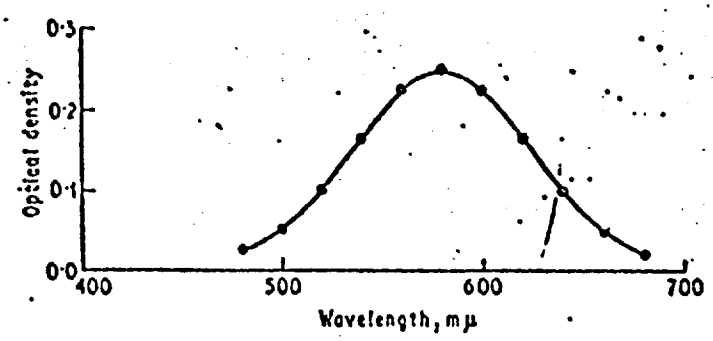



FIG. 2. Spectrum of the compound formed between BHT-alcohol and Gibbs reagent.

A sharp maximum was obtained at 580 mμ, at which wavelength there is little reagent or tissue "blank" absorption. The optical density at 580 mμ was proportional to the amount of BHT-alcohol added, up to 0·05 μmole (Fig. 3).

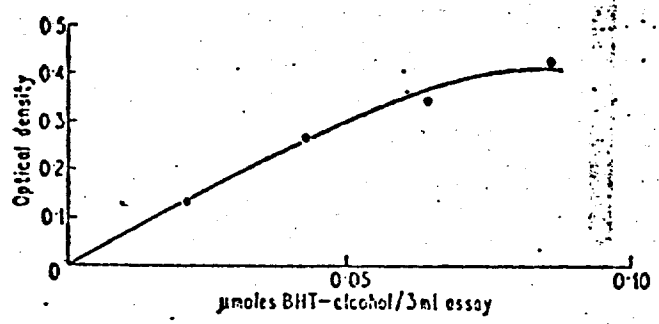



FIG. 3. Standard curve for BHT-alcohol estimation. Incubation mixtures were prepared as described in the Experimental section except for the omission of substrate and the inclusion of known amounts of BHT-alcohol dissolved in 2-methoxyethanol. Portions (3 ml) of these mixtures were taken through the standard estimation procedure.

*Assay and properties of rat-liver BHT oxidase*

The reaction rate was constant for 10–15 min and was proportional to the amount of enzyme added up to 0·4 ml microsomal suspension per 3 ml assay (Fig. 4). The enzyme was localized in the microsomal fraction (Fig. 5). The optimum pH was 7·4 (Fig. 6). $Mg^{2+}$ was

Table 1. *Cofactor requirements for BHT oxidase*

| Omissions from incubation system | Reaction rate (μmoles/hr/g liver) |
|---|---|
| None | 0·43 |
| $MgCl_2$ | 0·44 |
| NADP | <0·03 |
| Glucose 6-phosphate | <0·03 |
| None | 0·59 |
| Nicotinamide | 0·37 |
| NADP | <0·03 |
| Glucose 6-phosphate | <0·03 |
| Glucose 6-phosphate dehydrogenase | 0·04 |

BHT-oxidase activity was determined by the standard procedure (see Experimental section) except for the omission of the relevant component as stated.

Table 2. *Requirement of BHT oxidase for $O_2$*

| Procedure | | Activity |
|---|---|---|
| Pre-incubation | Incubation | (μmoles/hr/g liver) |
| 5 min $N_2$ | 10 min air | 0·68 |
| 5 min $N_2$ | 10 min $N_2$ | 0·03 |

Aliquots of the standard incubation mixture, without substrate were placed in the bodies of Warburg flasks and substrate in the side-arms. The flasks were gassed with $N_2$ for 5 min while shaking in a bath at 37°, then substrate was tipped into the main compartment. Incubation proceeded with the flasks either sealed (gas phase $N_2$) or open to the atmosphere (gas phase air). Each value is the mean of two experiments.

not required by BHT oxidase, but omission of nicotinamide from the incubation medium reduced the activity and no activity was obtained in the absence of NADP or of the NADP-reducing system (Table 1) or if oxygen was excluded (Table 2). NADPH could not be

replaced by NADH (1·5 mM) nor did this concentration of NADH, in addition to NADPH, enhance the activity observed in the presence of NADPH alone. No detectable increase in enzyme activity was obtained by doubling the concentrations of any of the cofactors. The $K_m$ value for reduced NADP was $1{\cdot}0 \pm 0{\cdot}2 \times 10^{-5}$M (2)* and for BHT was $1{\cdot}9 \pm 0{\cdot}2 \times 10^{-4}$M (5). Substrate inhibition occurred above $2 \times 10^{-3}$M-BHT. The enzyme was competitively inhibited by SKF 525A (Fig. 7) with $K_i = 8{\cdot}6 \pm 1{\cdot}2 \times 10^{-6}$M (4). In females and in young males the activity was low, but in adult males much higher activities were found (Fig. 8).

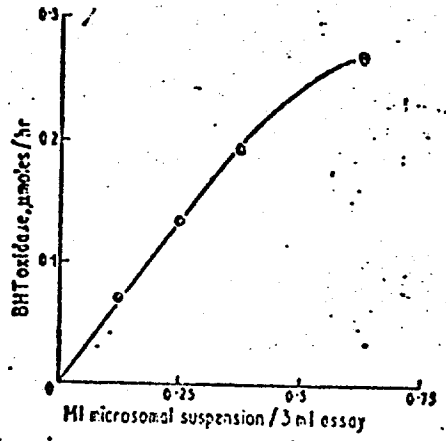



FIG. 4. Initial reaction rate at various enzyme concentrations. Enzyme activity was determined by the standard procedure except for variation of the enzyme concentration.

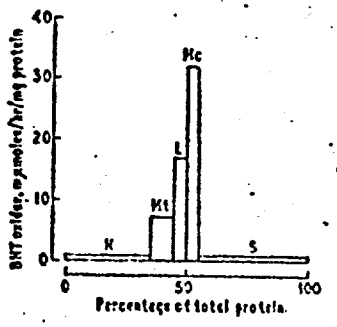



FIG. 5. Intracellular distribution of BHT oxidase. N, nuclei; $M_t$, mitochondria; L, lysosomes; $M_c$, microsomes; S, soluble fraction.

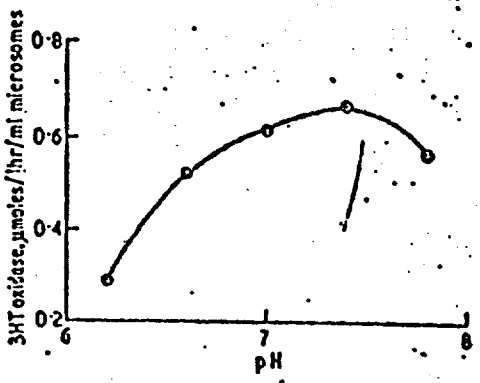



FIG. 6. Effect of pH on the activity of BHT oxidase. Enzyme incubations were buffered with potassium phosphate, 83·3 mM, at the indicated pH.

*Results are given as a mean ±SE followed by the number of estimations in brackets.

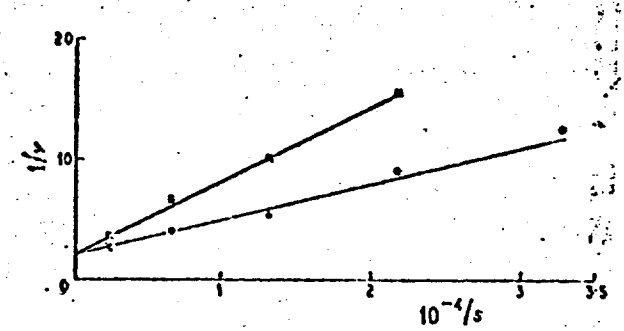



FIG. 7. Inhibition of BHT oxidase by SKF 525A. Enzyme activity was measured at various concentrations of BHT alone (○) and in the presence of $10^{-5}$M-SKF 525A (●). Results are plotted by the double-reciprocal plot of Lineweaver & Burk. $v$, μmoles/hr/g liver, $s$, concn (M) of BHT.

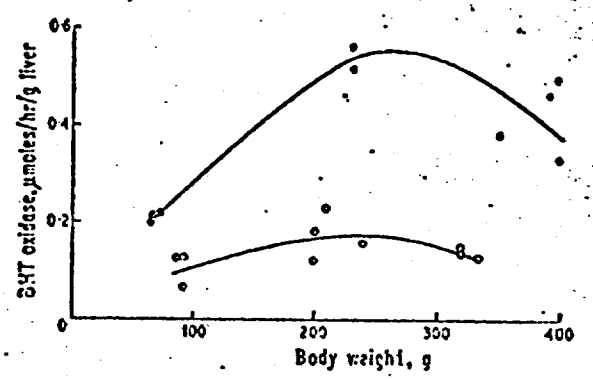



FIG. 8. Effect of age and sex on BHT oxidase. Liver microsomes were prepared from male (○) and female (O) rats of different ages and assayed by the standard procedure, as described in the Experimental section.

*Induction of rat-liver BHT oxidase*

Fig. 9 shows the effect of 5 daily oral doses of 500 mg BHT/kg given to female rats, in increasing the level of BHT oxidase and the return of enzyme activity to its original value when treatment was discontinued. The enzyme level in liver microsomes rose within 24 hr and continued to rise up to 5 days of treatment. The relative liver weight was unchanged at 24 hr but was raised at 48 hr. When treatment ceased, the enzyme level fell between 24 and 48 hr after the final dose and had returned to its starting value within 7 days. Relative liver weight dropped more slowly at first but reached the control level after 7 days' recovery. These changes were not accompanied by any significant alterations in the microsomal protein content of the liver (Fig. 9).

The enzyme induction and liver enlargement caused by BHT were prevented by treating the animal with actinomycin D. When 100 μg/kg of actinomycin D was administered with the dose of BHT and three further doses of actinomycin given at 4-hr intervals, there was no increase in BHT oxidase at 24-hr (Fig. 10a). When the BHT treatment was continued for 2 days and the daily actinomycin D dose was halved, relative liver weight was unchanged and only a slight increase in BHT oxidase occurred [Fig. 10(b).]

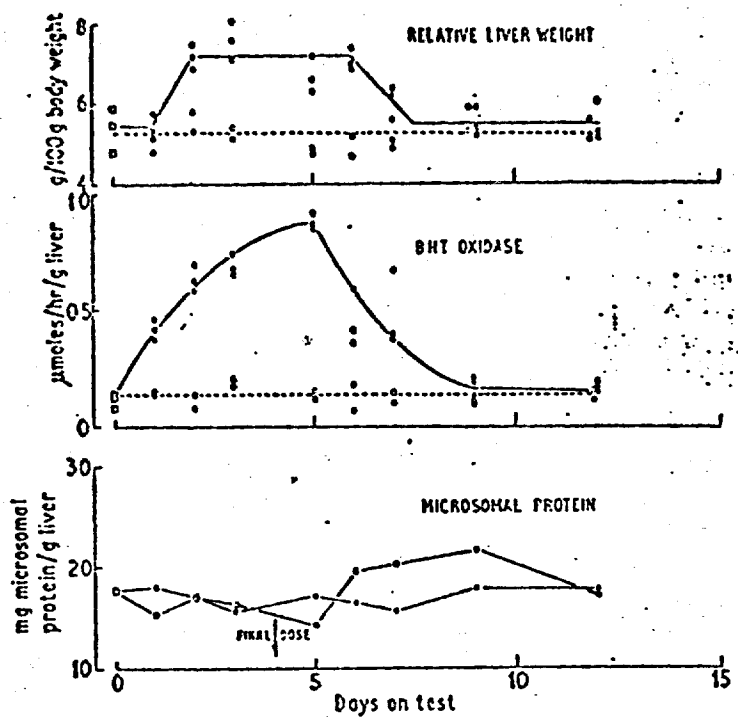



FIG. 9. Effect of BHT treatment on BHT oxidase. Female rats were given 500 mg BHT/kg daily on days 0–4. Measurements were made of the relative liver weight, liver microsomal protein and liver-microsomal BHT-oxidase activity of rats killed after 0, 1, 2, 3 and 5 doses and on 1, 2, 4 and 7 days after the fifth and final dose. □, untreated; ⊖, BHT-treated; ○, arachis oil controls. For relative liver weight and enzyme activity each point represents one animal; for microsomal protein each point represents two arachis oil (○), three BHT-treated (⊖) or three untreated (□) rats.

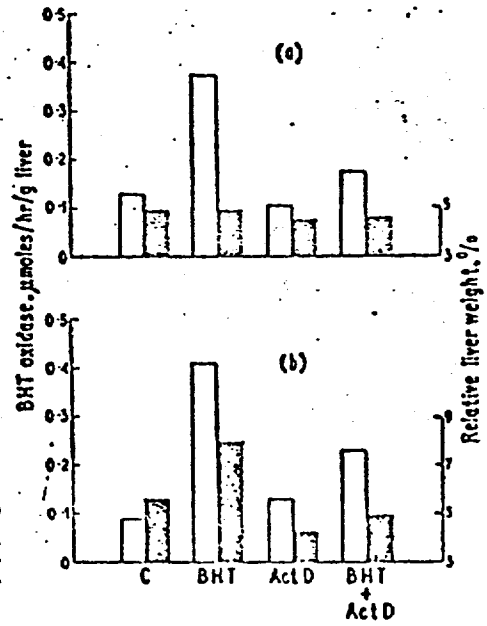



FIG. 10. Prevention of enzyme induction by actinomycin D.
(a)—Female rats were given a single oral dose of BHT (500 mg/kg) followed by intraperitoneal doses of actinomycin D (100 µg/kg) at 0, 4, 8 and 12 hr. They were killed at 24 hr and the relative liver weight and liver-microsomal BHT oxidase determined.
(b)—Female rats were given oral doses of BHT (500 mg/kg) at 0 and 24 hr and of actinomycin D (100 µg/kg) at 0, 12, 24 and 36 hr. They were killed at 48 hr and the relative liver weight and liver-microsomal BHT oxidase determined.
C, control; Act D, actinomycin D. Each bar represents the mean of two experiments. Shaded zones denote relative liver weight and unshaded zones BHT oxidase.

## DISCUSSION

Previous investigations of the urinary metabolites of BHT by Daere (1961), Agaki & Aoki (1962), Aoki (1962) and Wright *et al.* (1965) have indicated that oxidation of the 4-methyl group constitutes a major metabolic pathway of this antioxidant in the rabbit and rat. The present results show that the rat liver is capable of carrying out the first stage in this metabolic pathway and that the primary attack on the singularly non-reactive BHT molecule is not dependent on extrahepatic enzymes or gut flora. Furthermore, the enzyme which carries out this reaction has the properties of a typical processing enzyme in its requirement for NADPH and $O_2$, its neutral pH optimum, its localization in the microsomal fraction and its inhibition by SKF 525A.

The rise in BHT-oxidase activity following treatment of rats with BHT closely paralleled the rise previously observed by Gilbert & Golberg (1965) in the activities of hexobarbitone oxidase, aminopyrine demethylase and nitroanisole demethylase. As with hexobarbitone oxidase, the rise in BHT-oxidase activity preceded the rise in relative liver weight. The ability of actinomycin D to prevent the rise in BHT-oxidase activity indicates that this rise is due to a *de novo* synthesis of active protein, rather than the activation of a precursor, in common with the induction of other processing enzymes by hydrocarbons and phenobarbitone (Gelboin & Blackburn, 1963; Orrenius & Ernster, 1964).

The effect of BHT did however differ from that of DDT and of phenobarbitone in that there was no significant rise in the microsomal protein content of the liver. In several experiments with BHT it has been found that total microsomal protein was not increased by more than about 10%. This finding contrasts with the striking proliferation of endoplasmic reticulum brought about by repeated doses of phenobarbitone (Remmer & Merker, 1963; Herdson, Garvin & Jennings, 1964 a, b) and by DDT (Fouts & Rogers, 1965; Ortega, 1966) and suggests that BHT may elicit the adaptive rise in processing enzymes without causing a gross increase of smooth endoplasmic reticulum*. Such a response would be surprising because of the non-specific nature of the enzyme-induction effect caused by BHT. Of six enzymes studied in the present investigation and by Gilbert & Golberg (1965), only one (codeine *O*-demethylase) was unaffected while five apparently distinct enzyme activities were markedly raised, namely aniline (aromatic ring) hydroxylase, hexobarbitone (cyclohexene ring) oxidase, BHT (methyl side-chain) hydroxylase, nitroanisole *O*-demethylase and aminopyrine *N*-demethylase.

It is instructive to compare the BHT-oxidizing potential of the liver, as revealed by our *in vitro* studies, with the overall rate of BHT metabolism in rats given large or small daily doses of BHT. We have shown previously (Gilbert & Golberg, 1965) that when rats are given high daily doses of BHT, an initial period of adaptation is followed by a "plateau" in which no further changes occur in relative liver weight, processing-enzyme activity or tissue BHT levels in spite of continued daily dosing. Under these conditions a state of equilibrium has been attained in that the rate of excretion of BHT must equal its rate of absorption. The rate of reaction found in the microsomes of untreated rats, i.e. 0·15 $\mu$moles/hr/g liver, coupled with the relative liver weight of 5·3% gives a total metabolic potential of 42 mg/kg/day. This value should be increased to allow for incomplete recovery of activity in the microsomal fraction (see Fig. 5) and should be reduced to allow for the lower substrate concentration *in vivo* than that used *in vitro* (equivalent to 100 ppm) and to the lower concentrations of NADPH. The unmodified value may therefore be not far

**Note added in proof:* Proliferation of the endoplasmic reticulum in rats given high doses of BHT has been reported recently by B. P. Lane & C. S. Lieber (*Lab. Invest.* 1967, 16, 342).

removed from the true metabolizing potential of the animal and it is noteworthy that only when the dose exceeds this estimate of the metabolizing potential are the phenomena of liver enlargement and enzyme induction observed (Gilbert & Golberg, 1965). Similarly after 5 daily oral doses of 500 mg/kg the measured activity of 0·87 μmoles/hr/g liver with a relative liver weight of 7·2% corresponds to a metabolic potential of 330 mg/kg/day. This is consistent with the achievement by that time of a balance between the absorption and excretion of BHT, as indicated by the maintenance of a constant tissue level of BHT in spite of continued dosing (Gilbert & Golberg, 1965).

From these findings we conclude that the normal rat can metabolize appreciable doses of BHT and that as long as the BHT intake does not exceed this metabolizing capacity no changes are observed in the liver. A moderate excess of the BHT intake over the normal metabolic capacity elicits a response by the liver which involves overall enlargement and induction of processing enzymes. This results in an increase in the ability of the liver to metabolize BHT and it is probably this ability of the rat liver to adapt to a high BHT intake which enables the animal to tolerate doses of several hundred mg/kg daily for several months without showing any histological abnormalities in the liver (Karplyuk, 1959). Only at very high doses, when the BHT intake exceeds the metabolic capacity of even the adapted liver, does one find signs of incipient liver damage such as loss of glucose 6-phosphatase (Feuer *et al.* 1965).

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## La BHT-oxydase, enzyme des microsomes du foie dont la sécrétion est provoquée par le traitment de rats à l'hydroxytoluène butylé

**Résumé**—On a démontré qu'un enzyme du foie de rat est capable d'oxyder l'hydroxytoluène butylé (2,6-di-*tert*-butyl-4-méthylphénol ou BHT). Le produit de la réaction est le 2,6-di-*tert*-butyl-4-hydroxyméthylphénol (BHT alcool). On décrit un dosage quantitatif pour cet enzyme; l'enzyme requiert du NADPH et de l'$O_2$, le pH optimum se situe à 7,4, les ions $Mg^{2+}$ ne sont pas nécessaires et aucune activité n'est obtenue avec le NADH. L'enzyme est inhibé de façon compétitive par le SKF 525A.

Le traitement de rats femelles au BHT (500 mg/kg par jour pendant 5 jours) provoque une multiplication par six de l'activité de la BHT-oxydase/g de foie et une augmentation de 35% du poids relatif du foie, toutes deux empêchées par l'actinomycine D. Cette augmentation du pouvoir oxydant du BHT explique les changements de rétention du BHT dans les tissus, communiqués auparavant.

## BHT-Oxydase. Ein mikrosomales Leberenzym, induziert durch die Behandlung von Ratten mit butyliertem Hydroxytoluol

**Zusammenfassung**—Die Fähigkeit eines Enzyms der Rattenleber, butyliertes Hydroxytoluol (2,6-Di-*tert*-butyl-4-methylphenol; BHT) zu oxydieren, wurde nachgewiesen. Das Reaktionsprodukt war 2,6-Di-*tert*-butyl-4-hydroxymethylphenol (BHT-Alkohol). Eine quantitative Bestimmung dieses Enzyms wird beschrieben; das Enzym erfordert NADPH und $O_2$, der optimale pH-Wert ist 7,4, $Mg^{2+}$ wird nicht benötigt und mit NADH wird keine Aktivität erhalten. Das Enzym wird durch SKF 525A competitiv inhibiert.

Die Behandlung weiblicher Ratten mit BHT (500 mg/kg täglich, 5 Tage lang) verursachte eine sechsfache Erhöhung der Aktivität von BHT-Oxydase/g Leber und eine Zunahme des relativen Lebergewichts um 35%, was beides durch Aktinomyzin D verhindert werden konnte. Diese Erhöhung der BHT-Oxydierungskapazität erklärt früher mitgeteilte Änderungen der Zurückhaltung von BHT im Gewebe.

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# BIOLOGICAL ACTIVITY OF BUTYLOXYTOLUOL*
(Literature Survey)

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V. A Gor'kov, V. V. Disvetova, L. S. Yelseyenko, S. S. Matveyeva.

Department of the Kinetics of Chemical and Biological Processes (Head--Academician N. M. Emanuel') Institute of Chemical Physics, Academy of Sciences USSR, Moscow

The protection of food products (especially fats and those containing fats) is both an economic and hygienic problem, since, as a result of fat oxidation, their vitamin content is lost and toxic and carcinogenic oxidation products are formed. [11].

It is known that fatty oxidation is a chain reaction. The formation, extension and branding of chains occurs with the aid of free radicals formed during the reaction. Synthetic anti/oxidants inhibit the oxidation process by reacting with these radicals to form stable products, thus shortening the chains [22].

For example, in the reaction of a peroxided free radical with a phenol type anti/oxidant:

a stable phenoxyl radical and hydroperoxide are formed which decompose to stable oxygen-containing products [18]. Using the electron paramagnetic resonance [EPR] method, free radicals were observed in almost all redox steps of glycolysis, respiration and other metabolic processes of the living cell [22]. The determining role of free radicals has been established in the developmental mechanism of a series

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* Translator's note: BHT is the common U.S. usage.

of pathological states of living organisms; aging, stress, malignant growths, radiation sickness, etc. For active inclusion in these processes N. M. Emanuel' [15] proposed the use of low toxicity inhibitors of free radicals, for example, food antioxidants. One of the most widely distributed of this class of substances is butyloxiytoluol [BHT] -2, 6-di-tertfutyl-4-methyl phenol, the properties of which are surveyed in this article.

Depending on the manufacturer, BOT is known as: butyloxytoluol, BHT, ional, toponol, antioxidant AS-1, AS-3, or DVRS (DBPK) and dibunol. Pure BHT is a white, crystalline, odorless substance, almost insoluble in water and dissolves well in fats and certain organic solvents. The hydroxyl group of BHT is shielded by two tertiary butyl groups, guaranteeing the stability of the corresponding phenoxyl radical and a high anti/oxidant activity. [16].

As an industrial antioxidant BHT was first patented in 1940, and in 1953--1954, Dugan [35] and Kraykill [49] proposed its use in protecting nutrient fats from oxidation.

Currently, BHT with other antioxidants (butyloxianisol, prophylgallate, etc.) are widely used for stabilizing motor fuels, rubber, petroleum products, plastics, and are added to food fats, cosmetic creams, soaps, vitamins, antibiotics, medicines, animal feeds, etc. to protect these products from premature spoilage and loss of their beneficial qualities [16, 49]. The maximum level of BHT is established by law as 0.01--0.02% (by weight). The World Health Organization (WHO) suggests the maximum permissible dose for humans as 0.5 mg/Kg body weight daily. The actual human use level for BHT from all sources is about 0.05 mg/Kg, or 2-3 mg/day [16]. These figures can be used to compare the amount of BHT taken in by animals in tests of toxicity and other properties of BHT.

TABLE ON SIZE OF MTD, $LD_{50}$ and $LD_{100}$ OF BHT FOR VARIOUS ANIMAL SPECIES

## SIZE OF MTD, $LD_{50}$ AND $LD_{100}$ of BHT FOR VARIOUS ANIMAL SPECIES

| Animal Species | Dose (in mg/Kg) MTD (MPD) | $LD_{50}$ | $LD_{100}$ |
|---|---|---|---|
| Mouse | --- | 2,000 | 2,500 |
| Rat | 500 | 1,700-2,450 | 3,500 |
| Guinea Pig | --- | --- | 10,700 |
| Rabbit | --- | --- | 2,100-3,200 |
| Cat | --- | --- | 940-2,100 |
| Dog | 4,700 | --- | --- |

Toxicity of BHT. In the case of animal feeding, the antioxidant level is 100 times the maximum allowed levels for humans, but there shouldn't be any secondary effects [28]. Relevant toxicological studies of BHT were made by I. A. Karplyuk, Deichman, A. Ya, and Broytman, et al [2,10,33, 53,55]. The maximum tolerated dose [MTD]*, $LD_{50}$ and $LD_{100}$ were determined for various animal species as shown in the table. In these experiments BHT was administered to animals in an oil base via a stomach tube. In regular administration, the MTD for rabbits (5% in rations, 800 mg/Kg once/day) and mice (250-500 mg/Kg), [24, 26, 33, 34], and also for rats (5% in rations) was measured. Animal deaths were noted from the fourth-tenth day after initial administration. Denz, et al. [34]. believed cause of death to be the abrupt destruction of electrolyte balance, particularly, loss of K s from muscles and tissues. In these experiments in the first 24 hours, refusal of food occurred (at 1 gm BHT/day), then, in rabbits, emaciation developed, a diminution in the amount of extracellular water, increased Na and K (20%) (up to 10 times) excretion in the urine. Simultaneously 5 administrations to dogs in two weeks [33] of much larger BHT doses (1400 - 4700 mg/Kg) produced no toxic effects. In examining the effects on mice, rats and dogs of chronic administration in food of small amounts of BHT, most authors concluded 0.1% BHT in food produces no functional or

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* MPD

morphological changes when given for 2 years[23, 25, 29, 33, 38, 53]. The investigation of higher BHT levels led to some contradictory results. Thus, Deichman et al, in 1955 did not notice signs of intoxication in rats receiving 0.8% BHT in rations for two years; only at a level of 1% was some weight loss and increase in liver size noted. Other authors reported increased blood cholesterol levels five weeks after feeding with rations containing 0.5% BHT [29], growth rate inhibition (more marked in the first month and less evident in the second) [10] and increased liver sizes [25]. Johnson [46], at the same dietary BHT levels, did not notice changes in growth rate and blood cholesterol levels. Frovley [38], at a dietary BHT level of 0.3%, noticed 20% blood cholesterol level increase in rats 28 weeks after the start of the experiments (after 10 weeks such a rise did not appear). Johnson et al [45], noted a diminished growth rate and a 10-20% increase in the relative liver weight.

Note that these negative effects occur only when animals are fed a diet containing 20% fats.

In experiments using such high-fat diets, a group of Australian researchers [29, 23, 45, 57] spoke out against the use of BHT as a food antioxidant. A critique of these conclusions was given in the work of Johnson, published in 1965 [46], in which he noted the statistical unreliability of the above results and an insufficiently high fat level in the rations. A series of authors consider it hard to explain the increase in cholesterol level only from the influence of BHT at such a high dietary fat level. Also, animals eat food with such high fat contents unwillingly, especially if administered by stomach tube. This leads to growth and weight losses. At normal dietary fat and BHT levels in I. A. Karplyuk's [11] diet there was no disturbance of metabolic processes in the organism.

In view of weight differences in experimental animals and the amount of food they eat per day, it is hard to give corresponding values,but approximately one can state that at a safe dietary BHT level (0.1%), the rodent equivalent is 50-100 mg/Kg body weight per day [24]. The gap between maximum possible and actual BHT levels in the human diet is about 0.5-0.05 mg/Kg/day. This quantity is practically harmless.

Biological tests conducted in rats and mice over a two year period have shown a constant absence of teratogenic and mutagenic effects at a 1% dietary level and also at single

and repeated massive doses (to 1,000 mg/Kg at various periods before and after pregnancy [9, 20, 24, 33, 38, 46.]

All authors who conducted morphological examinations of tissues from animals receiving BHT found no changes in the organs. Persistent hematological indices (reversible) appeared in doses exceeding 0.1% in the diet.

The attention of researchers was drawn to increased liver weight in experimental animals. When rats are given 0.5% BHT in food (500 mg/Kg/day), within a day there occurs increased enzyme activity in the liver microsomal fraction, involving a change in rates of dimethylation, hydroxylation and oxidation of certain medicinal substances in vitro upon incubation with liver homogenates of control and experimental rats [25, 43]. Within two days, relative and absolute liver weights increased sharply. A parallel increase in urinary ascorbic acid excretion occurred, the BHT level in abdominal fat rose to 250 mg/Kg in females and to 162 mg/Kg in males. The maximum liver weight increased (to 130%) and increased enzyme activity (to 214%), and also urinary ascorbic acid excretion in rats was observed on the third day and remained constant until cessation of BHT administration. Simultaneously the BHT level in fat fell to 100 mg/Kg in both males and females and remained constant despite continuing BHT administration [42]. Liver weight increase and phenomena accompanying it were observed only after increased daily BHT doses (to about 25-75 mg/Kg/day).

At lower doses the BHT level in intestinal fat (mg/Kg) was about 0.75, and was 0.19 at 500 mg/Kg/day BUT doses [43]. In attempting to explain these phenomena, Gilbert, et al. [44] detected an enzyme oxidizing BHT to its hydroxymethyl derivative and identified the product itself. The enzyme is localized in the microsomal fraction and requires the presence of oxygen and $DPNH_2$ at an optimal pH of 7.4. Normal enzyme activity is 0.15 μmol/hr/gm of homogenate, and in rat liver subjected to 500 mg/Kg BHT doses for five days -- 0.87 μ mol/hr/gm. Actinomycin D is the enzyme inhibitor. The administration of the hydroxymethyl BHT derivative to rats in equimolar quantity does not, in itself, evoke the liver weight and enzyme activity increase, rather its metabolism and excretion from the organism occurs faster than BHT [44, 57]. On this basis the conclusion is that, in the experiments described above, BHT, and not its metabolites, accumulate unchanged [44].

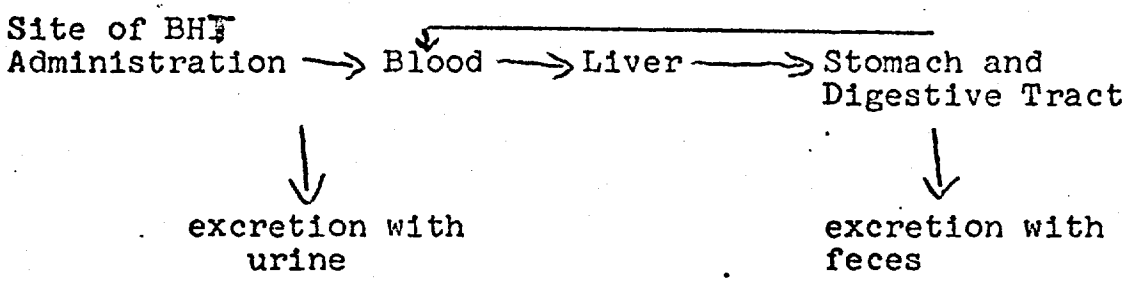
Some researchers [39, 44, 45] believe that in BHT-caused liver weight increase, the physiological response of the organism to introduced exogenic substances is comparable to muscular hypertrophy under appropriate stress. These authors conclude that BHT causes no pathomorphological changes in the liver. The benefit of such a conclusion is confirmed in the constant cholesterol phospholipid and free fatty acid levels in the liver and also in the reversibility of effect [25, 41].

All described phenomena are related to BHT dose, significant BHT level increases in human diets are consequently not dangerous as a dietary antioxidant [16]. BHT-induced liver enzyme activity is of special interest in connection with the detoxification of harmful substances, while increased urinary ascorbic acid excretion suggests itself as a criterion of non-toxic liver hyperfunctional increase [44].

BHT Metabolism and Excretion. One study [33] notes increased urinary sulfate and glucuronide excretion in rabbits subjected to 25 mg/Kg BHT doses intravenously in leathin emulsion. Dacre and Skage [19, 26] reported that in oral administration of BHT to rabbits in 0.5-0.8 g/Kg doses, 54-61% of the dose is excreted in the urine. Excretion is almost complete by the 4-5th day, leaving an unknown residue which suggests that BHT or its metabolites can accumulate in the body. However, such accumulation has not been observed. During prolonged dietary administration, most of the BHT is concentrated in fatty tissue in comparison to the parenchymatous organs. For each level of BHT concentration in the food there is an equivalent significant concentration in fat which decreases after dietary BHT intake ceases. During the next 8-10 days half of the BHT is eliminated. At the very low dietary BHT levels, its concentration in animal fatty tissue is almost nil [38, 54]. Remaining unknown in the experiments of Dacre and Akage is what part of the administered BHT dose was explained by other authors [30, 31, 56]. Apparently, in addition to urinary excretion, a significant amount of BHT is eliminated with the feces. In fact, these studies showed that most of a $C^{14}$ labeled BHT sample was excreted in the feces as compared to the urine. In all, about 90% of the labelled substance was excreted within four days, and excretion ceased by the 8-10th day. There remained very little additional tissue radioactivity. When proportioned doses were given it was discovered that the tissue excretion rate for labelled substances was higher than the BHT excretion rate [30].

Excretion of significant tracer amounts with bile and feces in subcutaneous and intra-abdominal administration of labelled BHT leads to the establishment of an entero-hepatic cycle [51,52], according to the following diagram:

ENTERO-HEPATIC BHT CYCLE




There is a characteristically high initial rate for the entero-hepatic cycle: upon intravenous injection of labelled BHT in 0.3 mg/Kg doses, more than 90% of it is excreted via bile discharge within six hours [50]. After a short initial period (about 24 hours) an equilibrium is reached in which about 5%/hr. of the residual radioactives are discharged. The slow BHT excretion rate noted by the authors above are indicative of completion of a cycle rather than accumulation of BHT in tissues.

BHT metabolic investigations utilizing paper and thin layer chromatography of bile, urine, feces and their extracts, with further study of spectra, eluents and also back synthesis of possible metabolites suggested that the basic metabolic pathway of BHT in the body is the oxidation of the methyl group to the corresponding alcohol, aldehyde and acid with formation of conjugates of the oxidized form with glycine, acetylajcysteine, cystine, glucuronic and sulfuric acids [19, 21, 27]. Oxidation in the tert-butyl groups is met only in human BHT metabolism. In this case, BHT metabolism proceeds basically along the pathway of similarly formed metabolites [32]. It is true that species differences in BHT metabolism govern varying excretion rates of this compound. In man, labelled BHT is basically excreted in the urine [31]. Since unlabelled BHT was observed neither in urine nor bile, a significant metabolic rate

for BHT is suggested, not less than that for intravenously administered BHT excreted with bile [50].

Thus, the foregoing studies confirm the safe use of BHT as a food antioxidant.

In connection with the capacity of BHT, as with other antioxidants, to affect free-radical reactions in biological systems, this preparation is of interest both to chemists and biologists and, beginning in 1956, has been studied in the Department of Kinetics of Chemical and Biological Processes of the Institute of Chemical Physics, Academy of Sciences USSR.

Studies of BHT in mice with Erlich ascites carcinoma [4] in vivo and in human Hep-2 cell cultures, showed that BHT inhibits L-$C^{14}$ amino acid uptake into the RNA fraction. Because inhibition is dose dependent, a 75 mg/Kg BHT dosage causes a 30% inhibition of L-$C^{14}$ amino acid inclusion, while a 115 mg/Kg dose produces 65% inhibition. A BHT concentration of 200 mg/Kg suppressed RNA inclusion of these aminoacids completely. DNA biosynthesis is not inhibited by this. Thus, RNA synthesis is more sensitive to BHT effects than is protein biosynthesis as a whole. In studying the effects of BHT on free radical concentration in the spleen and other organs of leucotic mice, and also the concentration of free radicals during the development of other tumors, the EPR method showed that BHT inhibits the completion of free radical reactions in such pathological states as the development of malignant tumors. [18]. Since it is established that the EPR signal is, to a significant degree, activated by succino-dehydrogenase activity, a decrease in the number of free radicals by BHT speaks of its effect on this enzyme. [13].

One of the developmental characteristics of a pathological process in the organism (especially carcinogenic) is a change in the antioxidative activity (AOA) in fatty tissue [12]. In single BHT administrations at the maximum tolerated dose of 100 mg/Kg and in 30 mg/Kg doses the liver lipid AOA in animal tests increased. This increase went parallel with decreased free radical activity. Upon further increase of the total BHI dose, AOA declined. Thus, upon six-fold BHT administration in 50 mg/Kg doses, the liver lipid AOA was 380 mg/l/hr.

Therefore, BHT could be classed as having antitumor properties, and this has been confirmed in mice with spontaneous and transplanted leucoses [15,17].

Since, in some preparations, antitumor action is combined with anticarcinogenic, so is the anticarcinogenic effect of BHT in an experiment on 150 male rats [14]. The animals were fed a carcinogin (0.06 dimethylaminoazobenzol in standard rations). Liver cancer appeared in 60% of the animals within 90 days, and in 90% within 120 days. Upon addition to the food, beside the carcinogen, of 0.3% BHT (by weight) in corn oil, all rats (80 common Wistar and 14 special August) developed no tumors during the 12-15 months observation period. No precancerous changes were found in rats killed at different stages after BHT and carcinogen addition. It was found that BHT decreased the amount of carcinogen found in liver protein more than five-fold.

It has been stated previously that in case of radiation damage BHT, as with other inhibitors of free-radical processes, can react with free radicals, formed during irradiation and by such means, diminish the severity of radiation damage. An analogous mechanism possible is the inhibition by phenols of biochemical redox processes, in which an important role is played by the intermediately formed free-radical compound. Such participation of BHT in the metabolism of substances can be used as a radioprotector as well as for anticancer effect. The multiple properties of BHT are explained by its capacity to change the course of free radical reactions [18].

To explain BHT's radioprotective properties, the relationship between survival time of lethally irradiated mice (BALB line) and amount of BHT administered as a radioprotectant was studied [3]. This relationship is of an extremal character. Upon increase in the BHT dose, the number of animals surviving after irradiation, began to increase. The greatest animal viability was observed with a 30-60 mg/Kg BHT dose. At further increases of the BHT dose animal viability after irradiation declined, and at a 100 mg/Kg dose there was almost no protective effect, rather the percentage of deaths as compared to those among controls, (that is, irradiated animals who received no BHT) increased [3]. It was shown also that BOT displays antitumor action over a dose range from 60 mg/Kg to the MPD. A lesser quantity of this compound has radioprotective properties and stimulates cell multiplication [3].

From the many theoretical and experimental studies discussed above, the clinic of the First City Clinical Hospital imeni N. I. Pirogav (Moscow), after a decision of the Pharmacological Committee of the Ministry of Public Health USSR conducted a three-pronged study of BHT. To test for suppression of regeneration, (in this case inhibition of hematopoiesis and various radiation and trophic skin diseases) they use small doses -- up to 2 gm/day [5,6]. For increased sensitivity to radiation therapy average doses were used (2-3 g) and for cancer therapy (urinary) higher doses were used -- more than 3 g. [17, 8].

All the data given illustrates BHT's broad spectrum biological effect, its low toxicity, the expediency of its further use not only in industrial, food processing, but also medical and biological applications.

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# A Rapid Gas Chromatographic Method for the Determination of BHA and BHT in Vegetable Oils

KENNETH T. HARTMAN and LUCIEN C. ROSE, Frito-Lay Research Department, Irving, Texas 75060


## Abstract

A gas liquid chromatographic (GLC) technique has been developed which requires about 20 min for the determination of BHA and BHT in vegetable oils. This method involves the addition of an internal standard to a weighed portion of the oil, dilution of the mixture with carbon disulfide, and injection into the Gas Chromatograph. BHA and BHT are isolated from the nonvolatile vegetable oil by using a short precolumn located in the sample port block of the gas chromatograph. Up to 35 consecutive sample injections per day have been made on the same precolumn with no appreciable effect on the accuracy of the determination. The precolumn is cleaned at the end of each day's operation. The clean precolumn is allowed to equilibrate to sample port block temperature overnight for the following day's analysis. Identification of BHA and BHT can be confirmed with a second GLC column which reverses the elution order of these compounds. Soybean, cottonseed, corn and peanut oils fortified with 20, 60 and 100 ppm each of BHA and BHT showed a recovery range of 97% to 104%.


## Introduction

Butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are frequently added to oils and fats to retard oxidation.

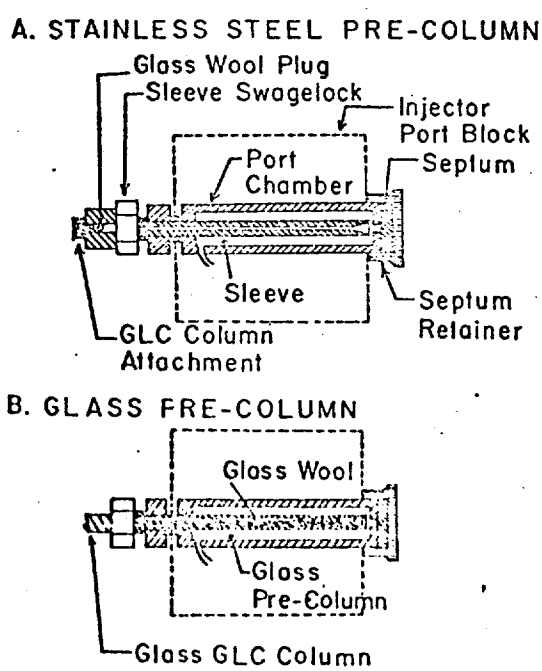


FIG. 1. Diagram of precolumns. Part A: stainless steel precolumn. The stainless steel sleeve is inserted into the port chamber and is secured with Swagelok fittings. Part B: glass precolumn. The first 5 in. of the GLC column (precolumn) inserts into the port chamber. The column is secured with a Swagelok nut and Hewlett-Packard "O" rings.

Current methods for BHA and BHT in vegetable oils, fats and fatty foods include extraction of the antioxidant from the product followed by gas chromatographic analysis of the extracts (1–4) or colorimetric determination from their steam distillates (5,6). Product extraction followed by thin layer chromatography (TLC) offers still another approach to the determination of BHA and BHT in oils (7). All of these procedures require several hours of analytical time.

A gas liquid chromatographic (GLC) procedure has been developed which requires about 20 min and exhibits good accuracy and reproducibility.

## Experimental Procedure

### Apparatus

A Hewlett-Packard F and M Model 5750 Gas Chromatograph equipped with dual flame ionization detectors was used.

### GLC Column Preparation

Column packing materials were prepared using the procedure of Burke and Holswade (8). Packed columns were conditioned for 72 hr with 50 ml/min helium flow through the system. Columns packed

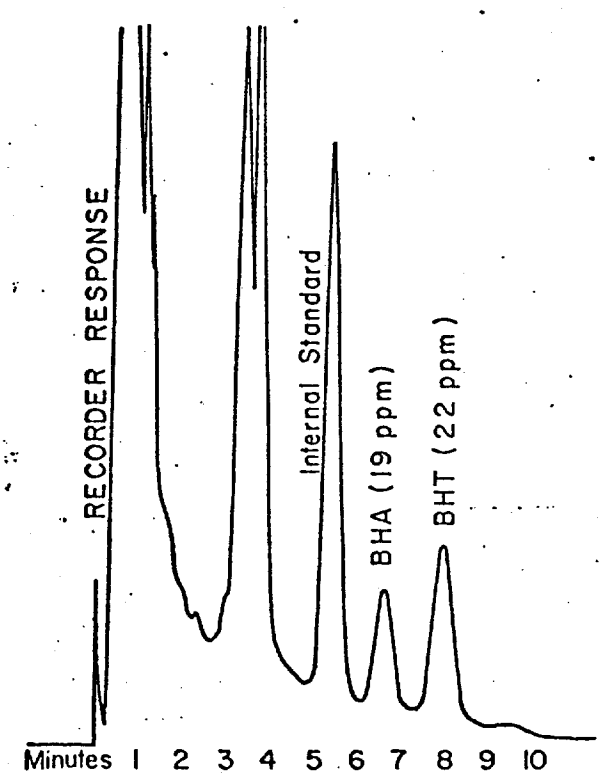


FIG. 2. A 6 ft × 4.5 mm i.d. aluminum column packed with 10% DC 200 on 80–100 mesh Gas Chrom Q, column: 160 C; detector: 250 C; injector port: 250 C; flow-rate: 50 ml/min helium; sensitivity: 4 × $10^{-13}$ amp. full scale deflection; sample: cottonseed oil fortified with BHA and BHT; sample size injected: 6.0 μl containing 0.100 g oil/ml.

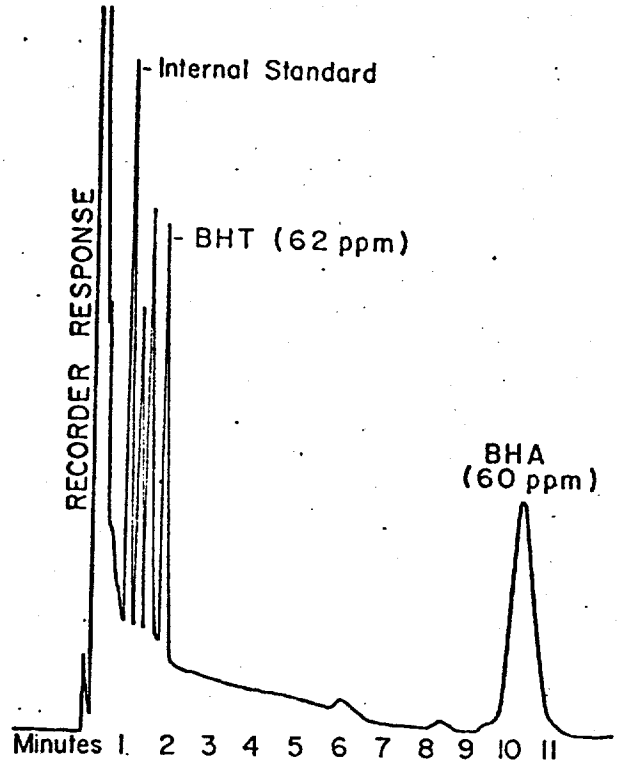


FIG. 3. A 4 ft × 4.5 mm i.d. aluminum column packed with 10% Carbowax 20 M on 80–100 mesh Gas Chrom Q, column: 190 C; remaining conditions same as Figure 2; sample: cottonseed oil fortified with BHA and BHT; sample size injected: 5.0 μl containing 0.100 g oil/ml.

with 10% DC 200 or 10% Carbowax 20 M were conditioned at 250 C and 200 C, respectively.

### GLC Precolumn Preparation

Two GLC precolumn variations were tested: First, a 6 ft × 4.5 mm i.d. (¼ in. o.d.) aluminum column packed with 10% DC 200 (12,500 cst.) on 80 to 100 mesh Gas Chrom Q. The GLC column was attached to a 1/16 in. i.d. stainless steel sleeve placed in the injector port block of the gas chromatograph. (This sleeve is a standard injector port block sleeve for the Hewlett Packard F and M Model 5750 Gas Chromatograph.) A small, loosely packed wad of siliconized glass wool (about ¼ in. in length) was placed in the outlet end of the injector port sleeve. When the sample aliquot was injected into the gas chromatograph, the nonvolatile oil remained in the stainless steel sleeve. The sleeve was removed from the gas chromatograph at the end of each day's analysis. The interior walls were washed with chloroform and the siliconized glass wool plug was replaced with a new plug. The sleeve was then installed in the gas chromatograph and allowed to equilibrate at operating temperature overnight (Fig. 1, Part A). Second, a 6 ft × 4 mm i.d. glass column packed with 10% DC 200 (12,500 cst.) on 80 to 100 mesh Gas Chrom Q. The first 4 in. of the column were packed with siliconized glass wool (Analab JU6). An on-column injection was used to introduce the sample into the gas chromatograph. The nonvolatile oil remained in the siliconized glass wool precolumn. At the end of each day's analysis, the glass wool containing the oil was replaced with new siliconized glass wool and the column was allowed to equilibrate overnight at operating temperature (Fig. 1, Part B).

TABLE I

Recovery of BHA and BHT From Oils

| Oil | Antioxidant added, ppm | Antioxidant Recovered: On aluminum column, % | Antioxidant Recovered: On glass column, % |
|---|---|---|---|
| **BHA as Antioxidant** | | | |
| Soybean | 20.0 | 101.4 | 101. |
| | 59.9 | 104.4 | 102. |
| | 99.8 | 102.1 | 100. |
| Cottonseed | 20.0 | 97.4 | 98. |
| | 59.9 | 97.4 | 99. |
| | 99.8 | 98.0 | 100. |
| Corn | 20.0 | 100.4 | 98. |
| | 59.9 | 100.9 | 102. |
| | 99.8 | 101.3 | 101. |
| Peanut | 20.0 | 103.5 | 97. |
| | 59.9 | 103.4 | 100. |
| | 99.8 | 103.2 | 98. |
| Average (12) | | 101.1 | 100. |
| Std. Deviation | | 2.83 | 1. |
| **BHT as Antioxidant** | | | |
| Soybean | 20.7 | 99.2 | 101. |
| | 62.2 | 101.1 | 101. |
| | 103.7 | 100.2 | 99. |
| Cottonseed | 20.7 | 103.8 | 101. |
| | 62.2 | 100.8 | 102. |
| | 103.7 | 99.3 | 100. |
| Corn | 20.7 | 99.1 | 100. |
| | 62.2 | 98.4 | 98. |
| | 103.7 | 100.4 | 101. |
| Peanut | 20.7 | 101.9 | 98. |
| | 62.2 | 102.7 | 100. |
| | 103.7 | 99.9 | 99. |
| Average (12) | | 100.6 | 100. |
| Std. Deviation | | 1.53 | 1. |

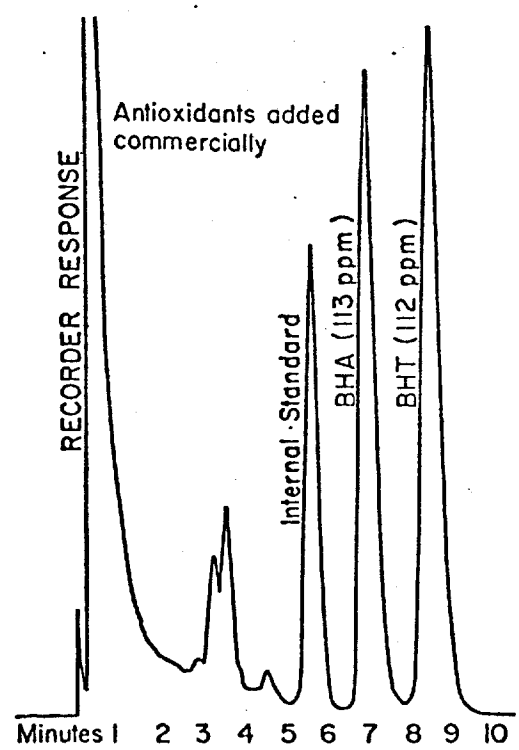


FIG. 4. A 6 ft × 4 mm i.d. glass column pa 10% DC 200 on 80–100 mesh GAS Chrom Q, colum remaining conditions same as Figure 2; sample: sp mercially prepared oil; sample size injected: 5. taining 0.100 g oil/ml.

TABLE II

Reproducibility Data for Method Tests

| Oil | Aluminum (GLC) column ppm antioxidants found | | | Glass (GLC) column ppm antioxidants found | | |
|---|---|---|---|---|---|---|
| | BHA | BHT | Total | BHA | BHT | Total |
| Special[a] | 114.4 | 113.9 | 228.3 | 114.5 | 111.4 | 225.9 |
| commercially | 115.1 | 114.1 | 229.2 | 113.4 | 111.5 | 224.9 |
| prepared oil | 113.5 | 111.1 | 224.6 | 115.7 | 112.6 | 228.3 |
| Average (3 tests) total antioxidants | | | 227.4 ppm | | | 226.4 ppm |
| Reproducibility range (total antioxidants) | | | | 98.8% to 100.8% | | 99.3% to 100.8% |
| Standard deviation | | | | 1.99 | | 1.43 |
| Peanut oil[a] | 86.4 | 69.3 | 155.7 | 81.5 | 68.3 | 149.8 |
| | 85.7 | 67.1 | 152.8 | 83.2 | 69.2 | 152.4 |
| | 86.8 | 69.4 | 156.2 | 83.7 | 67.3 | 151.0 |
| Average (3 tests) total antioxidants | | | 154.9 ppm | | | 151.1 ppm |
| Reproducibility range (total antioxidants) | | | | 98.6% to 100.8% | | 99.1% to 100.9% |
| Standard deviation | | | | 1.50 | | 1.06 |

[a] Commercial oils containing BHA and BHT at levels intended to be 0.02% for the specially prepared oil and 0.015% for the peanut oil.

### Gas Chromatograph Conditions

Temperatures were the following: column, 160 C; detector, 250 C; and injector port block, 250 C. Gas Flows were: helium (carrier gas), 50 ml/min; hydrogen, 40 ml/min; and compressed air, 450 ml/min. Sensitivity was as follows: range, 10; and attenuator, 2. The chart speed was ½ in./min.

### Calibration of Flame Ionization Detector

Five standard solutions were prepared containing 2, 5, 8, 10 and 12 μg/ml each of BHA (Eastman) and BHT (Eastman) in carbon disulfide (Baker's Analyzed Reagent Grade). Each of the standard solutions also contained 6 μg/ml methyl undecanoate (Applied Science 99+ Purity) which served as an internal standard.

Each solution was injected into the gas chromatograph and the flame ionization detector calibrated using the procedure of Sawardeker and Sloneker (9):

$$K = \frac{\text{Peak area for antioxidant/peak area of internal std.}}{\text{Wt of antioxidant/wt of Internal Std.}}$$

### Assay Procedure

The oil, 2.50 g, was weighed into a 25 ml glass stoppered volumetric flask, and 5.00 ml of internal standard solution containing 30.0 μg/ml of methyl undecanoate in carbon disulfide were added. The mixture was made to volume with carbon disulfide. Three to seven microliters of the mixture were injected into the gas chromatograph.

The detector calibration technique of Sawardeker and Sloneker (9) was modified to determine the quantities of BHA and BHT present in the oil by converting the calculations to yield ppm of antioxidant as follows:

$$\text{Ppm Antioxidant} = \frac{\text{Wt of Internal Std. } (\mu g) \times \text{peak area of antioxidant}}{\text{Sample wt (g)} \times \text{appropriate K value} \times \text{peak area of Internal Std.}}$$

### Method Accuracy and Reproducibility

Soybean, cottonseed, corn and peanut oils were fortified at 0, 20, 60 and 100 ppm each of BHA and BHT.

A commercially prepared oil and peanut oil each containing BHA and BHT were also tested.

### Results and Discussion

The GLC columns used for quantitation of BHA and BHT were either glass or aluminum 6 ft columns packed with 10% DC 200 (12,500 cst.) on 80 to 100 mesh Gas Chrom Q and fitted with the appropriate precolumns. Recovery results for BHA and BHT in fortified soybean, cottonseed, corn and peanut oils ranged from 97% to 104% (See Table I). Determination of BHA and BHT in peanut oil and a commercially prepared oil showed a reproducibility range of 98.6% to 100.9% (Table II).

The 6 ft DC 200 columns provided a rapid elution with baseline resolution for the Internal Standard (methyl undecanoate), BHA and BHT (Fig. 2 and 4). Results obtained with both the 6 ft 10% DC 200 glass column (4 mm i.d.) and the 6 ft 10% DC 200 aluminum column (4.5 mm i.d.) showed close agreement, indicating that either precolumn variation was adequate for the determination of BHA and BHT in vegetable oils (Tables I and II). Detector calibration resulted in a linear response over the range of 0 to 120 ppm each of BHA and BHT.

In actual practice BHA and BHT could be detected as low as 2 ppm. However, quantitative accuracy fell off rapidly below the 5 ppm level.

The elution order of BHA and BHT was reversed by using a 4 ft × 4 mm i.d. glass column with a 4 in. siliconized glass wool precolumn or a 4 ft × 4.5 mm i.d. aluminum column with the stainless steel precolumn described. Both columns were packed with 10% Carbowax 20 M on 80 to 100 mesh Gas Chrom Q and operated under the same conditions as the 10% DC 200 columns, except column temperature, which was 190 C. The change in elution order with these columns provided a means of confirming the identification of BHA and BHT (Fig. 2, and 3).

Samples of soybean, cottonseed, corn and peanut oils which contained no BHA and BHT were chromatographed. Resulting chromatograms showed no significant peaks at the elution times of BHA and BHT with either the DC 200 or Carbowax 20 M GLC columns. The lack of interference peaks (compounds) indicates that reliable quantitative and qualitative results can be obtained using the outlined GLC systems. Consecutive injections of oil samples into the

precolumn GLC system showed that the technique provides reasonably long column life. Over 200 sample injections (up to 35 per day) resulted in no detectable differences in column efficiency or method accuracy.

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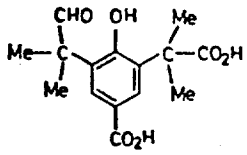
# LETTERS TO THE EDITOR

## The metabolism of butylated hydroxytoluene, (3,5-di-t-butyl-4-hydroxytoluene) in man

The urinary metabolites of [$^{14}$C]-3,5-di-t-butyl-4-hydroxytoluene (BHT) in man have been estimated by Daniel, Gage & others (1967), who found that over 50% of a 40 mg oral dose is excreted in the urine during the 24 h after dosing. The major metabolite (present to the extent of 35% of the dose) was later identified as a glucuronide of 4-carboxy-2-(1-carboxy-1-methylethyl)-6-(1-formyl-1-methylethyl)-



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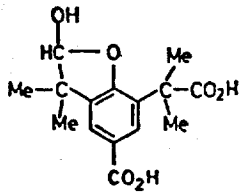

II

phenol (I) (Daniel, Gage & others, 1968). The agylcone was isolated as a hydrate of the dimethyl ester. Only 3% of the dose was eliminated as 3,5-di-t-butyl-4-hydroxybenzoic acid (BHT-COOH) and its glucuronide.

The oxidation of the t-butyl group has also been reported in rabbits given t-butylbenzene (Robinson & Williams, 1955) or BHT (Dacre, 1961). However, no t-butyl oxidation occurs with the t-butylcyclohexanones in the rat (Cheo, Elliot & Tao, 1967). Also Ladomery, Ryan & Wright (1967) were unable to demonstrate it with BHT in the rat and rabbit. It appears that when metabolic pathways other than oxidation of a t-butyl group are available such pathways predominate. It was therefore considered surprising that (I) was produced in such high yield in human urine. Investigations were made to verify the identity of this metabolite and determine the nature of the hydrate.

A group of 8 men each received 100 mg of BHT on two occasions with a 4 day interval. Urine was collected for 24 h after BHT administration and subjected to a work-up procedure identical to that employed by Daniel & others (1968), except that the DEAE cellulose column was omitted. The crude glucuronide gum was hydrolysed with 2M sodium hydroxide for 0·5 h and then continuously extracted with ether twice: once at pH 6 and again at pH 3. The metabolites present in the ether extracts were separated and identified using thin-layer chromatography (Holder, Ryan & others, 1970). Only BHT-COOH was detected in the first ether extract while the only component in the second extract to give a positive reaction with Gibb's reagent was identified as benzoylglycine (identified by infrared and nuclear magnetic resonance spectroscopy and melting point).

Subsequent urinary analyses were made in duplicate on the pooled 24 h urine of two adults each given 1·0 g of BHT. The urine was adjusted to pH 6 and extracted continuously with ether. A second extraction was made after further adjustment of the acidity to pH 2. Thin-layer chromatography of each extract showed the presence of a very polar compound which gave a positive test with naphthoresorcinol, and disappeared after $\beta$-glucuronidase or acid hydrolysis. Since the only compound present in significant amounts in the hydrolysed extract was BHT-COOH,

the polar component of the second extract was most probably BHT COOH ester glucuronide. The carboxylic acid and its ester glucuronide were the only major metabolites detected in human urine.

It is rather surprising that these were the only metabolites present. It was expected that if the metabolite (I) were the major metabolite in urine, it would have been easily detected and isolated. However three separate attempts, using doses comparable and greater than those used by Daniel & others (1968) failed. The evidence for structure (I) is incomplete as it is based almost entirely on accurate mass measurements of a molecular ion peak at m/e 322 for the dimethyl ester. The specific radioactivity of the metabolite (a radiolabelled compound had been fed) and details of its infrared spectrum were not given. Furthermore, although some nmr data are given no signals were assigned to the aldehydic and phenolic protons, and $D_2O$ exchange was not carried out. The absence of the aldehydic proton was ascribed to the formation of a stable hydrate, presumably the *gem*-diol of the aldehydic carbonyl. In general, diols obtained from aldehydes are unstable compounds only obtainable when the diol is stabilized by hydrogen bonding of the hydroxyl protons as with glyoxyllic acid, or by relief of dipole interactions as with trichloroacetaldehyde. For (I) there is no obvious driving force for such a reaction.

The cyclic hemiacetal (II) was considered as an alternative structure for Daniel's metabolite. However, the evidence presented is not sufficient since the nmr spectrum should show signals for one exchangeable hydroxyl proton and one methine proton. The signal at 4·6 τ (Daniel & others, 1968) could be assigned to the latter, but the absence of the hydroxyl signal remains. Furthermore since hemiacetals are usually too unstable to be isolated as such we consider structure (II) as unlikely.

In view of the foregoing, the occurrence of (I) as a BHT metabolite in human urine requires further verification. The postulated metabolite is unusual in that two alkyl groups have been completely oxidized to the carboxylic acid while the third is oxidized to the aldehyde. That oxidation of the t-butyl group proceeds beyond the alcohol is surprising, particularly in view of the results of Robinson & Williams (1955) who showed that t-butylbenzene is oxidized to 2-phenyl-2-methylpropanol only.

*Department of Pharmacy,*
*University of Sydney,*
*N.S.W.* 2006, *Australia.*

G. M. HOLDER
A. J. RYAN
T. R. WATSON
L. I. WIEBE

January 21, 1970

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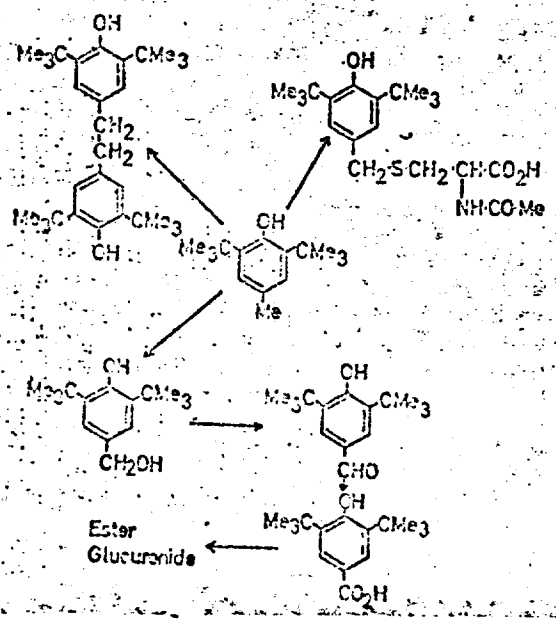
# The biliary metabolism of butylated hydroxytoluene (3,5-di-t-butyl-4-hydroxytoluene) and its derivatives in the rat

G. M. HOLDER, A. J. RYAN, T. R. WATSON AND L. I. WIEBE

*Department of Pharmacy, University of Sydney, Sydney, Australia*

The biliary metabolism of 3,5-di-t-butyl-4-hydroxybenzyl alcohol (BHT–$CH_2OH$), 3,5-di-t-butyl-4-hydroxybenzaldehyde (BHT–CHO) and 3,5-di-t-butyl-4-hydroxybenzoic acid (BHT–COOH) after parenteral administration has been examined in the rat and compared to that of 3,5-di-t-butyl-4-hydroxytoluene. Quantitative excretion and chemical examination of bile showed that in the enterohepatic circulation BHT–COOH or its ester glucuronide is the recirculating compound from the four compounds studied. Biliary excretion data are also presented for 1,2-bis(3,5-di-t-butyl-4-hydroxyphenyl)ethane.

The metabolism of the widely used antioxidant butylated hydroxytoluene (3,5-di-t-butyl-4-hydroxytoluene; BHT) has been reviewed by Hathway (1966). The enterohepatic circulation of its metabolites in the rat was suggested by Daniel & Gage (1965) and demonstrated by Ladomery, Ryan & Wright (1967a), who showed that the metabolites were rapidly excreted into the bile, and relatively slowly into the urine and faeces. The biliary metabolites (Fig. 1) in the rat were later shown to be 3,5-di-t-butyl-4-hydroxybenzaldehyde (BHT–CHO), 3,5-di-t-butyl-4-hydroxybenzyl alcohol (BHT–$CH_2OH$), 3,5-di-t-butyl-4-hydroxybenzoic acid (BHT–COOH) and 1,2-bis-(3,5-di-t-butyl-4-hydroxyphenyl)ethane (BB) (Ladomery & others, 1967b.)



These findings correlated with urinary metabolites of BHT in the rat and rabbit (Daere, 1960; Akagi & Aoki, 1962; Wright, Akintonwa & others, 1965). A previously unidentified metabolite in rat urine and bile was shown by Daniel, Gage & Jones (1968) to be *S*-(3,5-di-t-butyl-4-hydroxybenzyl)-*N*-acetylcysteine.

We now report on the biliary metabolism of BHT, BHT-$CH_2OH$, BHT-CHO, BHT-COOH and B-B in the rat. BHT-COOH was identified as the recirculating compound in the enterohepatic recirculation in the rat. Some additional urinary excretion data are reported.

## EXPERIMENTAL

### *Metabolic experiments*

White male Wistar rats (290–350 g), were given doses of [$^{14}$C]labelled BHT (or derivatives) in aqueous ethanol by intravenous or intraperitoneal injection. Urine and faeces were collected in metabolism cages fitted with all glass separating devices. Bile was collected through a biliary cannula for 6 to 8 h from rats under urethane anaesthesia.

### *Chromatography*

Silica gel G (E. Merck & Co.) was used for both qualitative and preparative thin-layer chromatography (TLC). Various solvent systems used were: A, chloroform–methanol (49:1); B, light petroleum b.p. 60–80°; C, light petroleum b.p. 60–80°–ether (2:1); D, n-propanol–chloroform (7:3); E, methanol–chloroform (2:3); F, n-butanol–ethanol–water (2:1:1). Visualization of BHT and its derivatives was achieved with Gibb's reagent (0·5% 2,6-dichlorobenzoquinone-4-chloroimine in ethanol); glucuronides were detected with naphthoresorcinol reagent (0·3% naphthoresorcinol in ethanol–phosphoric acid (5:1). Iodine vapour, and rhodamine (1% in ethanol) viewed under ultraviolet light (254 nm) were used as general reagents.

### *Methods*

Peroxide-free ether was used at all times. All reactions and *work-up* procedures were in a nitrogen atmosphere. Hydrolysis was effected by refluxing with N hydrochloric acid for 0·5 h, or by bacterial β-glucuronidase (Sigma type II) in 0·05M phosphate buffer, pH 7 at 37°.

Reverse isotope dilution analysis involved addition of unlabelled compound to the biological extract before work-up, and purification by preparative TLC. Other radiochemical assays were made on qualitative TLC chromatograms by counting after individual collection of the spots, suspension in scintillation fluid with Cab-O-Sil (Packard Instrument Co.).

"Free-phenols" refer to those compounds extractable from the biological sample by continuous ether partitioning at pH 6.

### *Materials*

The following compounds, used as TLC standards and for reverse isotope dilution, were prepared or obtained from the source indicated: 3,5-di-t-butyl-4-hydroxytoluene (BHT) and bis(3,5-di-t-butyl-4-hydroxybenzyl)ether (Shell Chemical Co., Sydney); 3,5-di-t-butyl-4-hydroxybenzyl alcohol (BHT-$CH_2OH$), 3,5-di-t-butyl-4-hydroxybenzaldehyde (BHT-CHO), 3,5-di-t-butyl-4-hydroxybenzoic acid (BHT-COOH) were

synthesized according to Ladomery & others (1957b); 1,2-bis(3,5-di-t-butyl-4-hydroxyphenyl)ethane (B-B), 3,3', 5,5'-tetra-t-butyl-4,4'-dihydroxystilbene (BHT-hydroxystilbene), 3,3',5,5'-tetra-t-butylstilbene-4,4'-quinone (BHT-stilbenequinone) were prepared according to Cook (1953).

The following [$^{14}$C] labelled compounds were used: [$^{14}$C]BHT randomly labelled in the t-butyl groups was obtained from New England Nuclear Corporation, Boston with specific activity $6{\cdot}55 \times 10^7$ d/min mg$^{-1}$ or $2{\cdot}85 \times 10^7$ d/min mg$^{-1}$.

[$^{14}$C]BHT-CHO was synthesized by bromine oxidation of [$^{14}$C]BHT (Coppinger & Campbell, 1953) and purified by preparative TLC (system A). The specific activity after diluent addition was $5{\cdot}58 \times 10^5$ d/min mg$^{-1}$.

[$^{14}$C]BHT-CH$_2$OH was prepared from [$^{14}$C]BHT-CHO by sodium borohydride reduction and purified by preparative TLC (Solvent A) giving a specific activity after diluent addition of $6{\cdot}19 \times 10^5$ d/min mg$^{-1}$.

[$^{14}$C]B-B. Areas corresponding to B-B, BHT-hydroxystilbene and BHT-stilbenequinone were isolated during preparative TLC used to isolate BHT-CHO. Sufficient BHT-stilbenequinone was isolated to reduce with lithium aluminium hydride in tetrahydrofuran (Bohn & Campbell, 1957). Preparative TLC (solvent A) of the product afforded [$^{14}$C]B-B with a specific activity after diluent addition of $5{\cdot}94 \times 10^5$ d/min mg$^{-1}$.

The aldehyde, alcohol, and diphenylethane were shown to be radiochemically pure by TLC in solvent systems A and B, A and C, and C respectively.

[$^{14}$C]BHT-COOH was prepared biosynthetically from 0–8 h bile of rats receiving an intravenous dose of [$^{14}$C]BHT. Diluent BHT-COOH was added to the bile and isolated by chloroform extraction after refluxing for 0·5 h with an equal volume of 2N hydrochloric acid. The crude acid was purified by preparative TLC using solvents D, E, and C, and the product, specific activity $5{\cdot}47 \times 10^5$ d/min mg$^{-1}$, was shown to be radiochemically pure (solvent systems A and F).

*Radioactivity measurements*

Radioactivity measurements were made using a Packard Scintillation Spectrometer, Model 3314, equipped for automatic external standardization. The scintillation fluid consisted of 0·15% PPO and 0·005% POPOP in 10% absolute ethanol in toluene. Aliquots of liquid specimens were counted directly using 10 ml of scintillation fluid; solution of dried finely ground faeces was achieved by digestion of an aliquot with Hyamine hydroxide solution (0·25 ml, Packard Instrument Co. and water 0·1 ml) at 37° overnight. Following addition of fluid, samples were stored in the dark for 24 h before counting.

## RESULTS

The hourly excretion of [$^{14}$C]labelled biliary metabolites of BHT, BHT-CH$_2$OH, BHT-CHO, BHT-COOH and B-B for 6 h after single intravenous doses is shown in Table 1. Table 2 shows the 2 hrly excretion of the same compounds (excluding B-B) for 8 h after single intraperitoneal doses. Both sets of data show that these compounds are rapidly absorbed, metabolized and excreted in the bile. While the rate of biliary excretion after dosing with the carboxylic acid is greater after intravenous injection, the bulk being excreted in the first hour, the total percentage excreted appears to be independent of the parenteral administration route chosen. By contrast, the total biliary excretion after BHT or BHT-CHO administration is rather less after intra-

Table 1. *Hourly biliary excretion of radioactive metabolites (% dose) during 6 h following single intravenous doses of BHT and related compounds*

| Time (h) after dosing | Compound BHT* (100 μg) | BHT-$CH_2OH$ (102 μg) | BHT-CHO (100 μg) | BHT-COOH (112 μg) | B-B (109 μg) |
|---|---|---|---|---|---|
| 1 | 46·6 ± 7·5 | 39·4 ± 8·0 | 63·1 ± 12·4 | 78·3 ± 9·8 | 26·5 ± 7·6 |
| 2 | 25·0 ± 8·0 | 12·1 ± 3·6 | 16·1 ± 4·8 | 1·9 ± 0·1 | 10·5 ± 3·6 |
| 3 | 12·6 ± 2·6 | 8·6 ± 5·7 | 4·2 ± 2·4 | 0·5 ± 0·1 | |
| 4 | 4·7 ± 1·8 | 4·2 ± 1·4 | 2·7 ± 1·9 | 0·2 | 3·3 ± 1·0 |
| 5 | 2·9 ± 0·6 | 2·6 ± 0·7 | 1·0 ± 0·7 | 0·1 | |
| 6 | 1·9 ± 0·4 | 2·1 ± 0·4 | 0·9 ± 1·2 | 0·1 | |
| Totals | 93·7 ± 11·6 | 69·2 ± 4·4 | 88·0 ± 4·5 | 80·8 ± 9·8 | 40·3 ± 5·8 |

* From Ladomery & others (1967a).
Results are the mean values for six rats except for BHT–CHO for which four animals were used. Standard deviations are shown.

peritoneal dosage than after intravenous dosage. The biliary excretion of the alcohol, about 70% of the administered dose, appears to be unaffected by its mode of administration.

The biliary excretion of radiolabelled metabolites of the alcohol, aldehyde and carboxylic acid was also examined for 6 h, 5 days after intraperitoneal administration of these compounds. Table 3 shows the percentage of dose excreted hourly. These results are comparable to those of Ladomery & others (1967a), who found that about 10% of an intraperitoneal dose of BHT was excreted over 6 h, 4 days after dosing.

The total overall excretion pattern of BHT and its related compounds excreted in urine and faeces for 5 days, and bile collected 120–126 h after dosing with a single intraperitoneal dose were examined (Table 4). Although biliary excretion for 120–126 h after intraperitoneal dosage of BHT was not measured, the results of Ladomery & others (1967a) indicate it would not be significantly different from that of its metabolites for this period. There are no significant differences in the total radioactivity excreted over 126 h after dosing, about 70% of the dosed radioactivity for each compound being recovered.

The chemical identity of the metabolites of BHT and related compounds in bile, urine and faeces was examined using reverse isotope dilution analyses, and thin-layer co-chromatography. The Rf values of some of the reference compounds are in Table 5. The predominant metabolite in all biological extracts was the carboxylic acid or its ester glucuronide.

Table 2. *Two hrly biliary excretion of radioactive metabolites (% dose) during 8 h after single i.p. doses of BHT and related compounds*

| Time (h) after dosing | Compound BHT* (100 μg) | BHT-$CH_2OH$ (200 μg) | BHT-CHO (200 μg) | BHT-COOH (100 μg) |
|---|---|---|---|---|
| 2 | 31·5 ± 6·4 | 57·[illegible] ([illegible]) | [illegible] ([illegible]) | [illegible] ([illegible]–71·5 |
| 4 | 14·1 ± 1·7 | 10·1 ([illegible]) | 12·9 ([illegible]–16·2) | [illegible] ([illegible]–18·6 |
| 6 | 6·2 ± [illegible] | 4·7 (4·3–5·0) | 5·6 ([illegible]) | [illegible] ([illegible]) |
| 8 | not measured | 2·2 (1·9–2·5) | 4·2 ([illegible]) | [illegible] ([illegible]–2·2) |
| Totals | 51·8 ± 6·6 | 74·[illegible] (72–77) | 61·3 ([illegible]) | [illegible] ([illegible]) |

* From Ladomery (1967a) shown as ± standard deviation for six animals.
( ) range of values for two rats.

TABLE 3. *Hourly biliary excretion of radioactive metabolites (% dose) during 6 h 5 days after single intraperitoneal doses of compounds related to BHT*

| Time (h) after dosing | BHT-$CH_2OH$ (102 μg; 8*) | Compound BHT-CHO (100 μg; 5) | BHT-COOH (105 μg; 4) |
|---|---|---|---|
| 121 | 2·3 ± 1·0† | 2·0 ± 1·2 | 2·3 ± 1·5 |
| 122 | 1·5 ± 0·5 | 1·1 ± 0·7 | |
| 123 | 1·2 ± 0·4 | 0·7 ± 0·5 | 2·3 ± 1·2 |
| 124 | 1·0 ± 0·1 | 0·5 ± 0·3 | |
| 125 | 0·9 ± 0·1 | 0·2 ± 0·1 | 2·0 ± 1·5 |
| 126 | 0·9 ± 0·2 | 0·3 ± 0·1 | |
| Totals | 7·9 ± 0·9 | 4·7 ± 2·2 | 7·2 ± 4·0 |

* No. of animals.
† ± Standard deviation.

Little attention was directed to the metabolic products of [$^{14}$C]BHT except in faeces collected during the 5 day interval after intraperitoneal dosage. Reverse isotope dilution experiments indicated that of the 50% of the dose appearing in the faeces, 40% was free BHT-COOH, and only 0·7% was unchanged BHT. Besides small amounts of nonpolar metabolites a further 40% of the excreted radioactivity was present as conjugated metabolites.

Only the biliary metabolites of [$^{14}$C]BHT-$CH_2OH$ were examined, the urinary and faecal excretion having been examined by Wright & others (1965). The carboxylic acid, BHT-COOH, was the major metabolite. Of the total radioactivity in 0–6 h bile, 14% was present as free phenols, BHT-COOH predominating, while the ester glucuronide of BHT-COOH was the major component in the remaining metabolites. The only other metabolite detected was a small amount of water-soluble material which was not examined further. Acid hydrolysis, followed by TLC showed that no unchanged BHT-$CH_2OH$ was present. Similar results were obtained after intravenous and intraperitoneal dosage.

In 120–126 h bile from rats receiving intraperitoneal doses of BHT-$CH_2OH$, traces of the alcohol, aldehyde and BHT-dimers were detected. However the major metabolites were BHT-COOH and its ester glucuronide.

Unchanged BHT-CHO was detected (2% of dose) in the 0–6 h bile of rats given [$^{14}$C]BHT-CHO (2230 μg) intravenously. Isotope dilution analysis indicated that

TABLE 4. *Total recovery of radioactive metabolites (% dose) of BHT and related compounds over 120 h after single intraperitoneal doses*

| | Compound | | | |
|---|---|---|---|---|
| | BHT (100 μg) | BHT-$CH_2OH$ (102 μg) | BHT-CHO (100 μg) | BHT-COOH (1000 μg) |
| Urine .. .. | 32·9 ± 1·0 (8) | [illegible] ± 6·9 (4 pairs) | 35·2 ± 7·3 (3 pairs) | 45·1 ± 7·9 (3 pairs) |
| Faeces .. .. | 36·9 ± 1·2 (8) | [illegible] ± 4·5 (4 pairs) | 29·7 ± 7·5 (3 pairs) | [illegible] (3 pairs) |
| Bile (120–126 h) .. | — | 7·9 ± 0·9 (8) | 4·7 ± 2·2 (5) | 7·2 ± 4·0 (4) |
| Total.. .. | 68·9 ± 1·4 | 71·3 ± 8·3 | 68·8 ± 10·8 | 69·7 |

± Standard deviation. ( ) No. of animals.
* Lacombe & others (1967a).

Table 5. *Rf values of BHT and its metabolites*

| Compound | Rf System A | Rf System B |
|---|---|---|
| BHT .. .. | 0·93 | 1·0 |
| BHT-$CH_2OH$ .. | 0·60 | 0·50 |
| BHT-CHO .. .. | 0·85 | 0·70 |
| BHT-COOH .. | 0·15 | 0·20 |
| BHT-Dimers .. | 0·95 | 0·90–0·95 |

free BHT-COOH (4% of dose) was present together with conjugated carboxylic acid (57% of dose). These quantities represent 7·8 and 84% of the biliary radioactivity. Acid hydrolysed urine of rats given intraperitoneal [$^{14}$C]BHT-CHO showed the presence of BHT-COOH (80% of urinary radioactivity) and unchanged aldehyde (1–2%) by TLC analysis. Faeces collected over the 5 days following dosage contained unchanged aldehyde (4% of dose by TLC analysis) while 82% of the radioactivity was present as the free carboxylic acid as demonstrated by reverse isotope dilution analysis.

Bile of rats receiving injections of [$^{14}$C]BHT-COOH revealed the presence of the free acid and its ester glucuronide as the only metabolites.

Solvent extraction of bile after intravenous injection of [$^{14}$C]B-B indicated that 3% of the dose (7% of biliary radioactivity) was present as free phenol. Ether extraction at low pH removed an additional 40% of metabolites. TLC investigations of the ether soluble fractions suggested that the metabolites were also dimers. No free BHT-COOH, or its ester glucuronide was detected in these experiments.

## DISCUSSION

The 6 h biliary excretion data following injection of low doses of BHT and related compounds indicate that with the exception of the diphenylethane (B-B) these compounds are all rapidly metabolized and excreted in the bile. Examination of the hourly excretion of radioactivity (Table 1) suggests that the rate limiting factor affecting elimination of these compounds or their metabolites into the bile is the rate of oxidation to the carboxylic acid. The total 6 h excretion of the alcohol is significantly ($P < 0·05$) less than that of the parent compound, the aldehyde and the acid. The latter compounds are about 80–90% eliminated in the bile in 6 h, compared to the 70% elimination of BHT-$CH_2OH$.

The small but significant difference in the total label excreted after intravenous injection of BHT and BHT-$CH_2OH$ may arise through pharmacokinetic factors, or through differences in the oxidation pathways followed by these two compounds.

The biliary excretion pattern after intraperitoneal injection of small doses of BHT and related compounds (Table 2) differs from that after intravenous dosage, suggesting that factors such as precipitation and absorption in the peritoneum, and storage of the drug in the body are important in the metabolism of these compounds after intraperitoneal dosage. However, quantitation of metabolites in bile 0–6, 120 h after intraperitoneal dosage with BHT-$CH_2OH$, BHT-CHO and BHT-COOH (Table 3) indicated no large differences in the amount of radioactivity excreted in a 6 h period. The metabolites found are not substantially different to those found by Ledoux et al. (1967a) after intraperitoneal administration of BHT.

In total excretion studies over 5 days with all compounds except B-B, substantial agreement was found with the results for BHT of Tye, Engel & Rapien (1965) (Table 4). For low doses of the compounds tested there were no significant differences in the totals of 5 days urinary and faecal excretion and 120–126 h biliary excretion, about 70% of the administered radioactivity being recovered over 5 days. There were individual differences in the ratio of urinary to faecal excretion over this period, faecal excretion appearing more important for doses of BHT-COOH. While these differences appear significant it is unwise to draw any conclusion from them because the rats used were not monitored for the constancy of their nutritional status and urinary pH.

The lack of differences in the overall excretion pattern and the biliary excretion of BHT and related compounds, strongly suggested that the compound or compounds responsible for the enterohepatic circulation of radioactivity following [$^{14}$C]BHT administration must be common to the metabolic pathways of all these compounds. Only BHT-COOH or its metabolites could reasonably be suspected since BHT-COOH and its ester glucuronide are known to be the main metabolic endproducts of BHT in bile (Ladomery & others, 1967a) and BHT-$CH_2OH$ in urine (Wright & others, 1965).

In verification of this, the major metabolite found in early bile after parenteral administration of BHT-$CH_2OH$, BHT-CHO and BHT-COOH was the free acid or ester glucuronide, the latter usually predominating. Examination of late bile after acid hydrolysis again showed the carboxylic acid to be the major radioactive component. Similar results were obtained by Ladomery & others (1967b) for late bile after BHT administration. Variations in the ratio of free acid to ester glucuronide in bile, urine and faeces are probably caused by differing degrees of hydrolysis in the large intestine, or during storage and work-up. As would be expected, less free carboxylic acid than the glucuronide ester is present in the bile. This conclusion verifies those of Daniel & others (1968) about the nature of the recirculating compound in BHT recirculation.

The biliary excretion of the diphenylethane (B-B) contrasts with that of the other compounds studied. Amongst the metabolites, BHT-COOH or other fragments were not recognized, the bulk of metabolites appearing as BHT dimers. This contrast with the metabolism of bis-(4-hydroxy-3,5-di-t-butylphenyl)methane(Ionox 220) and bis(3,5-di-t-butyl-4-hydroxybenzyl)ether(Ionox 201) (Hathway, 1966).

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*Research Section*

# A Re-examination of the Possible Teratogenic Effects of Butylated Hydroxytoluene (BHT) and its Effect on the Reproductive Capacity of the Mouse

A. R. JOHNSON*

*Commonwealth Antioxidant Research Project, University of Adelaide, Adelaide, Australia*

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**Abstract**—One possible cause for concern in the use of butylated hydroxytoluene (BHT; 3,5-di-*tert*-butyl-4-hydroxytoluene) as a food antioxidant is the reported occurrence of anophthalmia in the offspring of rats which had been fed on BHT.

The effect of dietary BHT and lard on the incidence of anophthalmia and on the reproductive capacity of the mouse has been studied. During the course of the experiment 7765 mice were born. Neither BHT nor lard induced anophthalmia. A dietary level of 0·5% BHT exercised some untoward effects on the reproductive capacity of the mouse, but at 0·1% of the diet BHT had no significant effect. The results indicate that under the conditions of the experiment and in the strains of mice employed, BHT is not a teratogenic agent.

## INTRODUCTION

Butylated hydroxytoluene (BHT; 3,5-di-*tert*-butyl-4-hydroxytoluene) is extensively used as an antioxidant in foodstuffs for human consumption. Doubts have been raised as to the advisability of the use of BHT (Brown, Johnson & O'Halloran, 1959; Day, Johnson, O'Halloran & Schwartz, 1959; Karplyuk, 1959; Johnson & Hewgill, 1961; Sporn & Schöbesch, 1961; Joint FAO/WHO Expert Committee on Food Additives, 1962; Golder, Ryan & Wright, 1962) and it has been deleted from the list of permitted food additives in Sweden, while Australia has not included it in its list of permitted antioxidants. In the United Kingdom, the Food Standards Committee (1963) recommended that BHT should be withdrawn from the list of permitted antioxidants. One possible reason for the ban is the reported occurrence of anophthalmia in the offspring of rats fed BHT (Brown *et al.* 1959). In this report, the workers were unable to differentiate between a genetic or dietary causal factor, but although the numbers involved were small, anophthalmia was only present when the parents had been fed BHT. In the present study the possible relationship between dietary intake of BHT and development of anophthalmia has been re-examined in the mouse. In addition a study has been made of the effects of BHT on the reproductive capacity of the mouse.

## EXPERIMENTAL

*Animals and experimental design.* The mice used in this work were an outbred random albino strain and the eyeless mice came from an anophthalmic strain which had been maintained for a number of generations in the Genetics Department, University of Adelaide.

*Present address: c/o Commonwealth Scientific and Industrial Research Organization, Division of Food Preservation, P.O. Box 43, Ryde, New South Wales, Australia.

Table 1. *Effect of BHT and lard on the reproductive capacity of the mouse*

| | | | First litter | | | | | | | Total reproductive lifespan | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | At birth | | | At 12 days | | | | At birth | | At 12 days | | |
| Dietary level of lard (%) | Dietary level of BHT (%) | No. of days on diet to birth | No. born | Pup weight (g) | Litter weight (g) | No. alive | Pup weight (g) | Litter weight (g) | Age at death (days) | No. born | Pup weight (g) | No. alive | Pup weight (g) | Total pup weight (g) |
| 10 | 0·0 | 63·8 | 6·75 | 1·56 | 10·45 | 6·42 | 5·78 | 37·0 | 495 | 52·8 | 1·66 | 48·2 | 6·26 | 296 |
| | 0·1 | 62·9 | 6·21 | 1·57 | 9·67 | 6·00 | 6·02 | 35·4 | 488 | 54·7 | 1·66 | 49·0 | 6·22 | 301 |
| | 0·5 | 73·8 | 5·75 | 1·61 | 9·62 | 5·21 | 5·23 | 29·5 | 479 | 51·2 | 1·62 | 44·5 | 5·52 | 247 |
| 20 | 0·0 | 56·5 | 6·46 | 1·59 | 10·43 | 6·29 | 5·51 | 35·9 | 481 | 58·0 | 1·66 | 51·6 | 6·17 | 316 |
| | 0·1 | 69·8 | 6·62 | 1·54 | 9·81 | 6·25 | 5·41 | 33·9 | 499 | 57·3 | 1·63 | 51·7 | 5·80 | 277 |
| | 0·5 | 91·7 | 5·87 | 1·57 | 9·45 | 5·21 | 5·16 | 27·5 | 543 | 50·3 | 1·67 | 44·0 | 5·64 | 246 |
| Pooled lard figures | 0·0 | 60·1 | 6·60 | 1·58 | 10·44 | 6·35 | 5·65 | 36·5 | 488 | 55·4 | 1·66 | 49·9 | 6·22 | 306 |
| | 0·1 | 66·4 | 6·42 | 1·56 | 9·74 | 6·13 | 5·72 | 34·4 | 494 | 56·0 | 1·64 | 50·4 | 6·01 | 299 |
| | 0·5 | 82·8 | 5·81 | 1·59 | 9·54 | 5·21 | 5·20 | 28·5 | 511 | 50·7 | 1·64 | 44·2 | 5·58 | 246 |
| LSD* 5% | | 18·0 | 0·83 | 0·09 | 1·32 | 0·84 | 0·36 | 2·9 | | 7·1 | 0·06 | 7·2 | 0·40 | 43 |
| LSD* 1% | | | | | | | | 4·0 | | | | | | |
| LSD* 0·1% | | | | | | | | 5·5 | | | | | | |

*Least significant differences for comparison of overall BHT means (n=15 degrees of freedom).

Litters of weanling mice were selected which contained at least 6 females and one male. The mice were identified by ear punch and females from 6 litters were distributed at random among 6 cages, with the restriction that no cage was to contain more than one female from a given litter. A male selected from each of the same 6 litters was placed in each cage, these males being mutually unrelated. Every cage thus contained one female, one male which was its sib, and 5 other unrelated females. Six diets were tested, these being assigned to the 6 cages at random. With 4 complete replications of this experimental design there were 144 females in 24 cages, i.e. 24 females to each diet. Two of the litters were born of mothers of the anophthalmic strain and the 12 females were eyeless. The blind females were included in order to exaggerate any possible relationship between BHT, anophthalmia, and a genetic factor.

*Diets.* The diets were prepared daily and food and water were supplied *ad lib.* The diets were the same as those described previously by Brown *et al* (1959) who incorporated levels of 0, 0·1 and 0·5% BHT in diets containing 10 and 20% lard.

*Conduct of experiment.* A few days prior to parturition, the pregnant females were transferred to individual nesting cages, where they continued to receive the same diet. When the litter was born the litter size and weight and the presence or absence of eyes was recorded on days 1, 5 and 12 after birth. The pups were killed on day 12, and the mother was allowed to rest in the nesting cage for 1 wk before being returned to her original mating cage. This process was repeated throughout the whole reproductive lifespan of the female. Towards the end of the experiment, when some of the females had ceased to bear young, the males were replaced by young males. In no case could the failure to produce young be ascribed to the male.

The experiment was terminated after approximately 18 months. When the females had ceased to produce young, they were killed and examined; the liver, kidneys, adrenal, spleen, and any organs or tissues showing unusual pathological features were examined by histopathological methods.

## RESULTS

During the course of the experiment 7765 mice were born, none of which showed any evidence of anophthalmia, even though 12 out of the 144 mothers were from the anophthalmic strain.

The effect of the various diets on the reproductive capacity of the mice is shown in Table 1. The total progeny in each litter, the corresponding total and mean pup weight, from each female were treated as units for analysis of variance. As the results of the first litters appeared to be somewhat different from the results obtained on all litters, the analysis was repeated on these separately.

The only significant differences appeared at the 0·5% dietary level of BHT when the figures for the two levels of dietary lard were pooled. BHT at the 0·5% level increased the length of time to the birth of the first litters and at 12 days after the birth the mean number of pups alive, the mean pup weight and the mean total weight of these litters was lower than the overall average. Over the whole reproductive lifespan the 0·5% dietary level of BHT resulted in lower means for pup weight and total litter weight 12 days after birth.

A dietary level of 0·1% BHT had no statistically significant effects on any of the parameters measured although a general trend reflecting the effects of the 0·5% dietary level was apparent.

The only consistent pathological abnormality was the development of adenocarcinomata; these occurred in various sites in the female mice. There was, however, no relationship between the development of these tumours and the dietary intake of BHT.

## DISCUSSION

When using experimental animals to test the safety of a food additive, the normal testing dose is a dietary level of at least 100 times the maximum amount likely to be present in human diet. In this experiment the normal testing dose is the 0·1 % dietary level of BHT. At this level BHT had no effect on the reproductive capacity of the mice, thus confirming our previous results on one reproductive cycle of the rat. Other workers (Telford, Woodruff & Linford, 1962) have in fact shown that BHT, and certain other antioxidants, have a definite prophylactic value in reducing the frequency of foetal resorptions in the rat. The 0·5% dietary level of BHT did exercise some untoward effects on the reproductive capacity of the mice, but this level is outside the normal testing dose and the results cannot be directly related to the safety or otherwise of BHT as a food additive.

The relatively high occurrence of adenocarcinomata is interesting as a reflection of the effect of an excessive breeding programme in experimental animals but is irrelevant, as there was no relationship between diet and incidence of tumours.

One possible cause for the concern on the use of BHT as an antioxidant in human foodstuffs is the reported development of anophthalmia in a number of young rats born from parents that had been fed on BHT (Brown *et al.* 1959). At that time the evidence was not sufficient either to ascribe a teratogenic effect to BHT, or to exclude that possibility. The present results show that, in the mouse, BHT does not affect foetal development. Although, for practical reasons this experiment was carried out on a species different from that on which the original observation was made, the results indicate that under the conditions of the experiment and in the strains of mice employed, BHT is not a teratogenic agent.

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## Nouvelle étude des effets tératologiques possibles du butylate d'hydroxytoluène (BHT), et étude de son effet sur la capacité de reproduction de la souris

Résumé—Une des causes possibles d'inquiétude dans l'emploi du butylate d'hydroxytoluène (BHT; 3,5-di-*tert*-butyl-4-hydroxytoluène) comme antioxydant alimentaire est l'apparition de cas d'anophtalmie dans la progéniture des rats auxquels on a fait absorber du BHT.

On a étudié l'effet du BHT alimentaire et du saindoux sur l'apparition de cas d'anophtalmie et sur la capacité reproductrice de la souris. 7765 souris sont nées au cours de l'expérience. Ni le BHT ni le saindoux n'ont produit d'anophtalmie. A la dose de 0,5% de BHT, il y a eu quelques effets nocifs sur la capacité reproductrice de la souris. Mais mélangé aux aliments dans la proportion de 0,1%, le BHT n'a eu aucun effet significatif. Les résultats indiquent que, dans les conditions de l'expérience et avec les lignées de souris utilisées, le BHT n'est pas un agent tératogène.

## Erneute Untersuchung der möglichen teratogenen Wirkung des Antioxydationsmittels butyliertes Hydroxytoluol (BHT) und seine Wirkung auf die Fortpflanzungsfähigkeit der Maus

Zusammenfassung—Ein möglicher Einwand gegen die Verwendung von butyliertem Hydroxytoluol (BHT; 3,5-Di-*tert*-butyl-4-hydroxytoluol) als Antioxydationsmittel für Futter ist das berichtete Auftreten von Anophthalmie bei den Nachkommen von Ratten, die Futter mit BHT-Zusatz erhielten.

Der Einfluss von verfüttertem BHT und Speck auf das Auftreten von Anophthalmie und auf die Fortpflanzungsfähigkeit der Maus wurde untersucht. Während der Beobachtungszeit wurden 7765 Mäuse geworfen. Weder BHT noch Speck erzeugte Anophthalmie. Ein Anteil von 0,5% BHT im Futter verursachte einige ungünstige Auswirkungen auf die Fortpflanzungsfähigkeit der Maus, ein Zusatz von 0,1% BHT hatte jedoch keine signifikante Wirkung. Die Ergebnisse lassen darauf schliessen, dass BHT unter den Versuchsbedingungen und bei den verwendeten Mäusearten keine teratogene Wirkung hat.

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# THE EFFECT OF THE ANTIOXIDANT BUTYLATED HYDROXY TOLUENE ON THE LIPID METABOLISM OF THE RAT

A. R. JOHNSON*† AND E. S. HOLDSWORTH‡

(*Commonwealth Antioxidant Research Project and Biochemistry Department, University of Adelaide, Adelaide, South Australia*)

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## Introduction

Butylated hydroxy toluene (BHT: 3,5-di-*tert*-butyl-4-hydroxy toluene) is extensively used as an antioxidant in foodstuffs for human consumption. Some years ago doubts were raised as to the advisability of the use of BHT[1-7]; but more recently the Joint FAO / WHO Experimental Committee on Food Additives[8] has given the same level of unconditional acceptance to BHT as to the antioxidant butylated hydroxyanisole (0-0·05 mg/Kg body weight overall daily intake). This change in attitude probably arose out of work published in an issue of Food and Cosmetics Toxicology, solely devoted to studies on BHT[9-15].

While there appears now to be no reason for concern regarding the use of BHT at the recommended levels in human diets, the reported[2,4] hypercholesteremic effect of relatively high dietary levels of BHT has now been confirmed[10,15]. A linear relationship between the amount of BHT in the diet of the rat and the level of serum cholesterol has been shown[4]. Hence any information obtained on a high dietary level of BHT should also be valid at lower levels of BHT. This principle has been used in the present studies concerning the mechanisms by which BHT affects the lipid metabolism of the rat and the enhanced toxicity of BHT when the fat level of the diet is raised[1].

## Experimental

Acetate-1-$^{14}$C (20 $\mu$c/100g body weight) was injected into adult rats which had been reared from weaning on diets that were or were not supplemented with 20% of BHT-free lard or of lard containing 0·5% dietary BHT. There were 6 male and 6 female rats in each group. The animals were sacrificed either 1 hr. or 3 hrs. after injection, and the expired $CO_2$ and the lipids from various tissues were isolated and chemically and radiochemically estimated. Growth and basal metabolic rats (BMR) studies had been previously carried out on the animals. The methods of animal management, composition of diets and general lipid methods have been described previously [1,2,4].

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† Present address: CSIRO Division of Food Preservation, P. O. Box No. 43, Ryde, N. S. W., Australia.

‡ Present address: Biochemistry Department, University of Tasmania, Hobart, Tasmania, Australia.

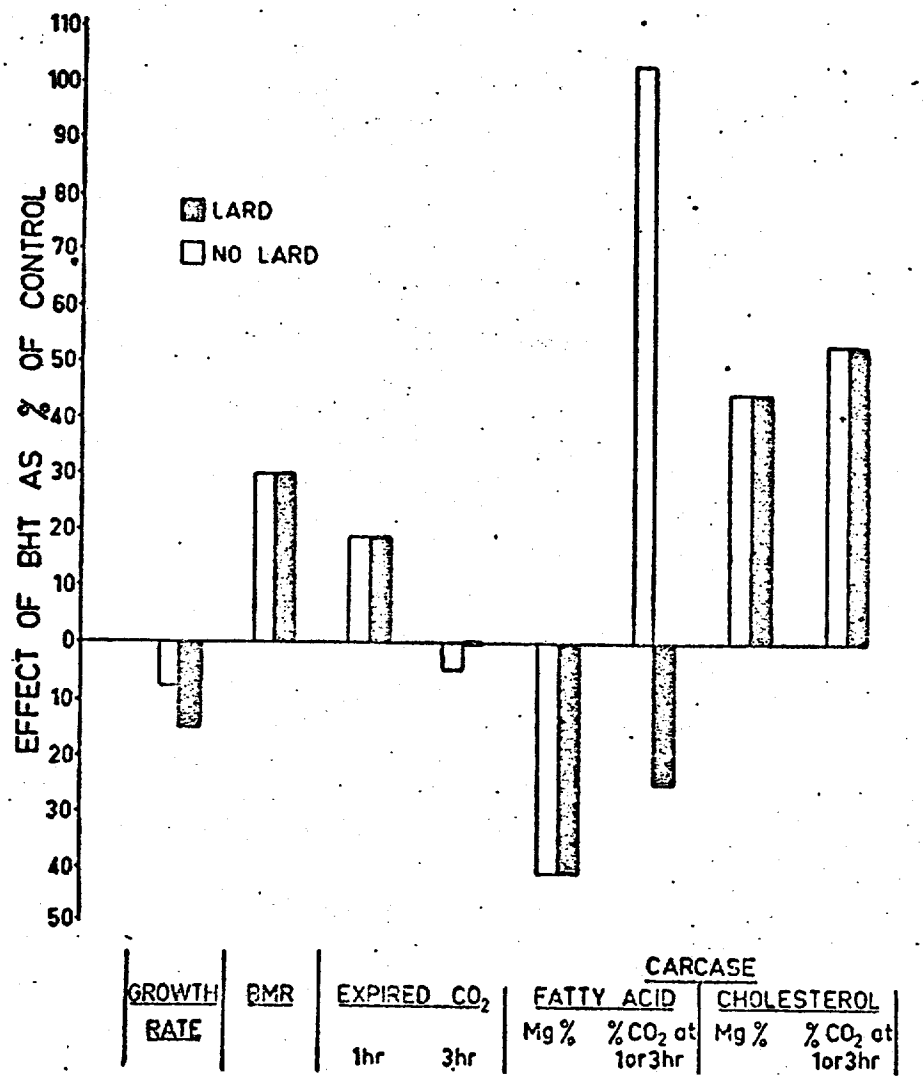


**Fig. 1.** The effect of BHT and lard on the growth rate and BMR of the rat and on the metabolism of acetate-1-$^{14}$C as expired $CO_2$ and as carcass lipids.

## Results and Discussion

The results have been summarized in Figures 1 and 2.

The horizontal line represents the normal values of each parameter for control rats not given BHT and provides a datum line, appropriate to whether the animals were fed lard or not. The vertical blocks represent the percentage changes from these normal values induced by 0·5% dietary BHT, blocks above the line indicating an increase and those below, a decrease.

BHT reduced the growth rate of the rats, and the earlier results were confirmed, in that the reduction in the growth rate was greater for the rats fed lard than for the rats not given lard.

A possible explanation for the lower growth rate of the animals fed BHT can be found in the increase in their BMR, with a consequent wastage of body tissue.

An increase in BMR was confirmed by the fact that there was an increase in the percentage of $^{14}C$ in the $CO_2$ expired during the first hour after the rats were fed BHT. After 3 hrs however, the animals fed BHT without lard supplement excreted $^{14}CO_2$ at a lower rate than their controls, the reason being that in the former an increased rate of incorporation of the label into carcass fatty acids reduced the amount of radioactively labelled $CO_2$ available for expiration. For the BHT diet with lard supplement the recovery of $^{14}C$-fatty acids from the animals was considerably less than those fed the BHT-free, non-supplemented diet, thus indicating a reduced rate of synthesis of carcass fatty acids in the first group. A reduction in the concentration of carcass fatty acids was found whether or not lard had been fed to the animals along with BHT. However, in the absence of the lard supplement the observed reduction in concentration of carcass fatty acids was accompanied by an increased rate of synthesis, so there must also have been an increase in the rate of breakdown of these fatty acids. In other words BHT must have produced an increase in the turnover rate of fatty acids in the animals not given the lard supplement.

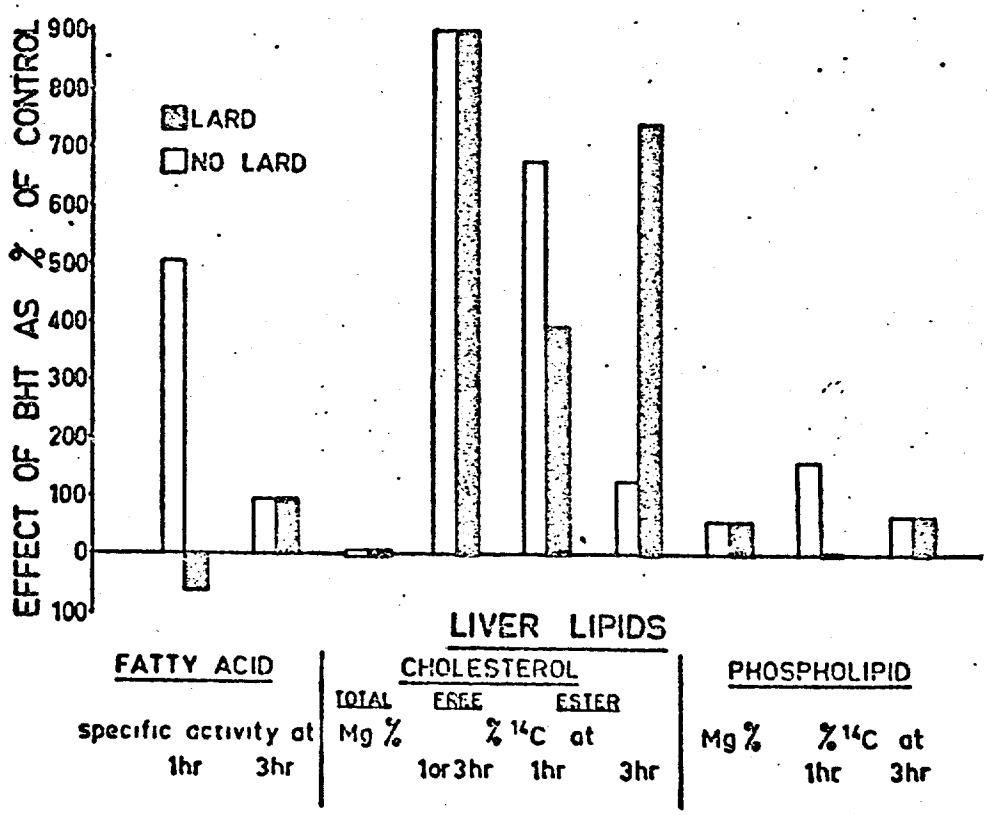


Fig. 2. The effect of BHT and lard on the concentration of rat liver lipids and on their biosynthesis from acetate-1-$^{14}C$

The concentration and rate of synthesis of carcass cholesterol were increased by BHT but the effect was not dependent upon the level of dietary lard.

The effect of dietary BHT, with and without lard on the incorporation of $^{14}C$ acetate into the liver lipids of the rat after 1 hr and after 3 hr, is shown in Figure 2.

The effect of BHT on the specific activity of the liver fatty acids after 1 hr differed according to the level of the dietary lard. After 3 hr this factor had ceased to operate and both groups showed the same increase over BHT-free controls.

The large initial increase in the specific activity of the liver fatty acids when BHT but no lard, was in the diet, and the subsequent large fall in the specific activity that had occurred within the first 3 hours, substantiates the suggestion that in rats dietary BHT induces rapid turnover of the fatty acids when a lard supplement is not given simultaneously.

Although BHT produced increases in the concentrations of both serum cholesterol and carcass cholesterol, and a very large increase in the rate of synthesis of liver free cholesterol in animals with and without dietary lard, there was no increase in the concentration of total liver cholesterol. The BHT-stimulated increase in cholesterol synthesis must therefore be accompanied by a faster removal of liver cholesterol to the serum and carcass, where it is deposited. It is interesting that in this case the concentration of liver cholesterol cannot be used as an index of the state of cholesterol metabolism, as the concentration gives no indication of the increased rate of synthesis of liver cholesterol.

The concentration of liver phospholipid was increased by BHT in animals with and without lard and the isotope work showed that the rate of synthesis of phospholipid was also increased. The liver must retain some of the phospholipid arising from the increased rate of synthesis.

The cholesterol esters and the phospholipids both contain fatty acid moieties, and similar effects of the interaction of lard, BHT, and the time factor are evident for both lipids. Where a lard supplement was not given, BHT increased the rates of synthesis of cholesterol ester and phospholipids although both increases were less at the third hour than at the first hour.

In contrast, with the lard-fed animals a reverse trend was apparent. Thus BHT with lard had no effect on the incorporation of $^{14}C$-acetate into phospolipid at 1 hour, but had increased the rate of synthesis of phospolipids by the third hour. Similarly, in the rats fed both lard and BHT the increase in $^{14}C$ incorporated into the cholesterol esters, and hence their rate of synthesis, was greater after 3 hours than after one hour.

In the lard fed animal, the slower initial rate of incorporation of $^{14}C$ into the cholesterol esters and phospholipids probably reflects the delayed incorporation into the liver fatty acids.

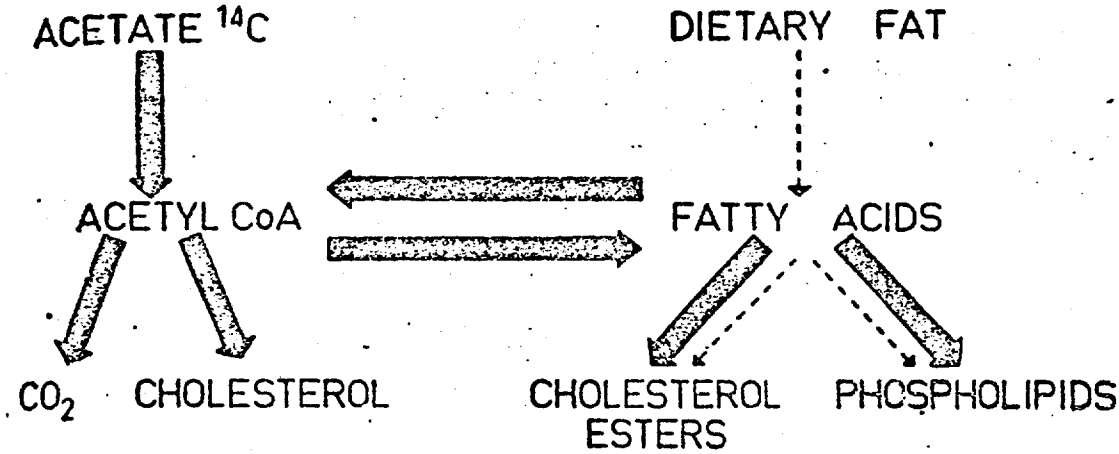


Fig. 3. The effect of BHT in the non-lard fed rat. The full black arrows indicate those reactions whose rates enhanced by the presence of BHT in the diet.

In Figures 3 and 4 an attempt is made to explain the action of BHT and why that action depends on the presence or absence of relatively large amounts of dietary fat.

Because of the absence of considerable amounts of dietary fat in the animal not fed lard, the main sources of tissue fatty acid are via synthetic pathways, and these synthetic pathways will be fully developed (Fig. 3). It is suggested that BHT by some means as yet unknown, increases the availability of acetyl coenzyme A, and so leads to an increased rate of production of $CO_2$ and cholesterol, the metabolic pathways of which are irreversible. This increased rate of production of $CO_2$ is shown by an increased basal metabolic rate and by increased wastage of tissue material, with consequent loss of weight. On the above hypothesis, BHT would also increase the rate of turnover of the fatty acids; hence, as the concentration of tissue fatty acids is lowered by BHT the catabolic pathways must predominate and therefore also produce loss of weight.

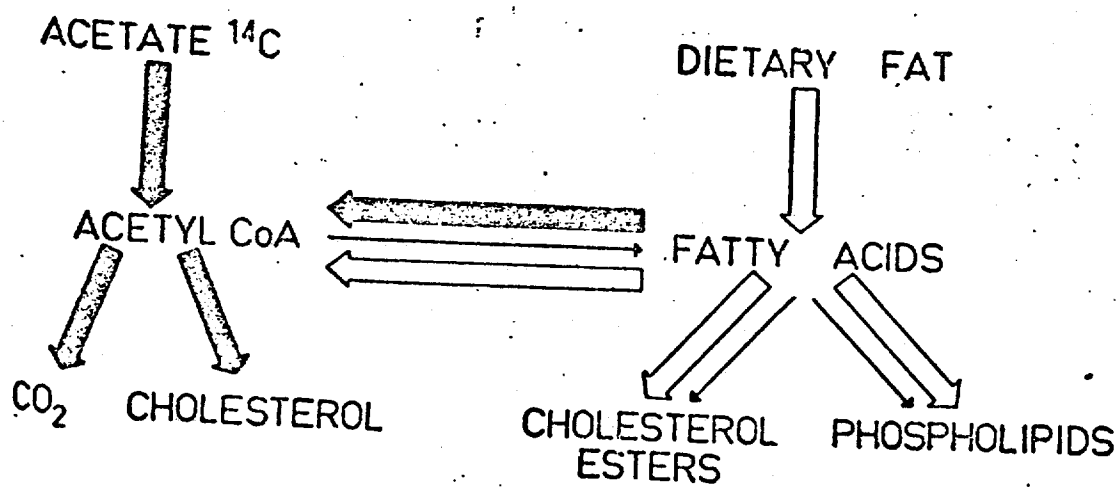


Fig. 4. The effect of BHT in the lard fed rat. The full black arrows indicate those reactions whose rates are enhanced by the presence of BHT in the diet and the open arrows those reactions whose rates are enhanced by the presence of lard in the diet.

In the lard-fed rat (Figure 4) there is a surplus of dietary fatty acids and the fatty acid composition of the cholesterol esters, phospholipids and the depot fat reflect those of the dietary fat. In addition, the catabolic pathways of fatty acids metabolism are enhanced, with a consequent depression of the contribution of the synthetic pathway from acetyl coenzyme A to fatty acid. When BHT is given in a lard-supplemented diet there could again be an increase in the availability of acetyl coenzyme A, leading to an increased excretion of $CO_2$ and increased production of cholesterol by the rat. However, because the synthesis of fatty acids from acetyl coenzyme A tends to be depressed by the presence of dietary fat the increased amounts of acetyl coenzyme A are not available for synthesis of fatty acids or of the fatty acid moiety of either cholesterol esters or phospholipids. Synthesis through the pathway cannot be suppressed entirely, however, and eventually some of the increased amounts of acetyl coenzyme A become available for the synthesis of fatty acids, thereby accounting for the differential effects with respect to time of sampling noted in the lard-fed rat.

A further implication is that, because the increased amounts of acetyl coenzyme A made available by the feeding of BHT cannot be fully utilized for fatty acid synthesis, BHT produces a greater reduction in growth rate of animals fed lard when compared with its effects on animals on a lard-free diet.

## Summary

The chronic toxicity effects of the antioxidant butylated hydroxy toluene (BHT) are accentuated by raising the fat level of the diet. This relationship has been investigated in the rat fed a diet containing 0·5% BHT in the presence or the absence of 20% lard by studying the effect of BHT on the metabolism of intraperitoneally administered acetate-1-$^{14}C$.

Irrespective of the presence or absence of dietary lard, BHT increased the basal metabolic rate, the amount of $^{14}CO_2$ expired during one hour after injection, the concentration of body cholesterol and the rate of synthesis of body and liver cholesterol, and reduced the total fatty acid content of the body.

In the animals fed BHT without lard, BHT increased the rate of synthesis and turnover of body and liver fatty acids and reduced the growth rate. In the animals fed BHT with lard, BHT reduced the rate of synthesis of body and liver fatty acids and reduced the growth rate to a greater extent than in the animals without dietary lard.

The effect of BHT on the synthesis of liver phospholipids and cholesterol esters was related to the time of slaughter after injection and to the inclusion or omission of dietary lard.

The results can be explained by postulating that BHT has a dual effect (a) by increasing the availability of acetyl coenzyme A and, (b) by increasing the catabolism of fatty acids. The first effect leads to an increased production of $CO_2$ and cholesterol, and in the animals without dietary lard an increased rate of synthesis of fatty acids which, together with the second proposed effect of BHT,

leads to an increased turnover of fatty acids. In the animals fed lard the exogenous fat depresses the synthesis of fatty acids from acetyl coenzyme A and the increased amounts of acetyl coenzyme A made available by the feeding of BHT cannot be fully utilized for fatty acid synthesis. Therefore BHT produces a greater reduction in growth rate of animals fed lard when compared with its effect on animals on a lard free diet.

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# TOXICOLOGICAL CHARACTERISTICS OF PHENOL OXIDANTS IN NUTRIENT FATS (ACUTE AND SUB-ACUTE EXPERIMENTS)

I. A. KARPLYUK

Department of Food Hygiene [Head -- Professor F. E. Budagyan] Central Institute of Post-Graduate Medicine
Moscow

Fats are highly valuable food products, giving quick energy which is the basis of the oxidative process. Oxidation at low temperatures is somewhat slow, but does not cease. Therefore, even in a refrigerator, fats can be kept no longer than six months. In the oxidation process fats lose their taste and nutrient properties and can exert harmful effects (I. A. Smorodintsev, 1947; Bur and Barues, 1943; Johnson, Perkins, Sugal and Kummerow, 1957, et. al.).

Not known completely is how fat oxidation products (peroxides, polymers) achieve their harmful effects or upon the oxidation of fats oxidize and thus activate the important portions of the fat, such as Vitamins A, B, E, K, pantothenic acid, pyridoxin, biotin, ascorbic acid, and other unsaturated fatty acids necessary to life (linoleic, linolenic, arachnidoic). Pathological phenomena developing during feeding of rats with a pelleted experimental diet can be explained insufficiently by the insufficiency of these nutrient substances in the diet (Quackenbusch, 1945; Greenberg and Frazer, 1953; Witting, Hishida, Johnson and Kummerow, 1957).

We also have data that deep oxidation of fats has a carcinogenic affect (Lavik and Baumann, 1941, 1943; Roffo, 1938). The thermic oxidation spectrum of fats is related to the spectrum of the carcinogenic derivatives of phenanthrene (Roffo, 1938).

One of the effective methods of protecting fats from oxidation is the addition of an antioxidant to the

fat. The greatest interest is in the phenol antioxidants, which are the most effective: butyoxianisol, butyoloxitoluol, and esters of gallic acid. In a series of countries these antioxidants are widely used in the food industry (Tollenaar and Vos, 1956).

At the present time in the Soviet Union the All Union Scientific Research Institute of the Meat Industry and the All Union Scientific Research Institute of Fats studied butyloxianisol (BHA), butyloxitoluol (BHT) and propyl gallate (PG) in frying fats and vegetable oils. Antioxidants are recommended for inclusion in quantities from 0.01--0.02%. At such a quantity butyloxianisol of BHA or BHT, fats increased their stability 3--5 times, and upon the addition of gallate, up to ten times (M. Emanuel', D. Kenoy, Yu. Lyaskovskaya, V. Piul'skaya, 1955, 1958). Organoleptic changes in fat by these antioxidants in the indicated quantities were not observed.

When these preparations were proposed for use in the food industry, the question of investigating their toxicology arose, A study on the toxicity of BHA was investigated by Lehmann, Fitzhugh, Helson and Woodard (1951), Wilder, Ostby and Gregory (1956), et. al., Takata, Nagata (1952), Deichmann, Clemmer, Rakoczy and Bianchine (1955) studied butyloxitoluol. The toxicity of gallates was determined by Boehm and Williams, (1943), Orten, Knyper and Smith (1948), Allen and De Eds (1951), Roy (1953), Sporn and Peretianu (1956). The work by the above mentioned authors was in the establishment of $LD_{50}$ of these preparations on mice, rats, guinea pigs, cats, the means of a method of establishing the dose differed with each author: one established it through sub-cutaneous administration, others by intra-peritoneal, a third through entreal methods; they gave solutions of differing concentration. It is understood that the methods of analyzing the results differed one from the other. In prolonged administration (one--three months) of large doses of phenol antioxidants (about 1% of feed weight), the authors noted interruption of growth and development in the animals. Propyl gallate at these concentrations leads, besides that, to a dimunition in the amount of hemoglobin in the blood and to pathological phenomena in the kidneys (the appearance of protein in the urine). BHA in concentrations of 0.25 grams per kg weight in dogs produces degenerative changes in the parencymatous tissue of the liver. Feeding gallates (oxtyl, dodecyl) to female rats in concentrations of 0.5% of feed weight led to cessation of lactation and obvious harmful effects

of milk on the young offspring. It is sufficient to note, that all authors in sub-acute tests used a very limited number of tests: animal weight, studies of the blood, and patho-morphological studies of the organs. Only Deichmann, et. al. (1955), studied the toxicity of BHT and determined the prothrombin time and measured blood pressure. It seems to us, that the number of tests used by these authors is insufficient to judge the functional state of the organism. In our experiments we tried to make a broader study of the various aspects of viability of the organism.

For comparative toxicological characteristics of these preparations (butyloxianisol, butyloxitoluol, propyl gallate) we set up acute tests on rats and mice. In the tests we took growing, healthy animals (males and females). Antioxidants were given in the morning, in an oil solution directly into the stomach via a metallic tube. The animals remained in cages after administration of the preparation and were kept under observation for 20 days. The animals received varying quantities of the studied preparations (7--8 doses). Each dose was given to eight rats and ten mice.

Through feeding of various doses of the studied preparations to the animals, we established the toxicity of these substances from a minimum toxic dose to absolute lethality. Then, in conducting toxocological integration according to Berens, (N. S. Progdine, 1957; V. S. Shadurskaya, 1953), we constructed a death curve and, according to it, determined the $LD_{50}$ of all three antioxidants for rats and mice. Results of the tests are presented in Table 1. From the obtained data it is evident that the toxicity of these preparations are very close to each other and that phenol antioxidants are comparatively non-toxic.

TABLE 1

RESULTS OF DETERMINING TOXIC AND LETHAL

DOSES OF THE STUDIED ANTIOXIDANTS

1. Antioxidant

2. Rats

3. Mice

4. Minimum toxic dose in mg/kg of body weight

5. $LD_{50}$ mg/kg body weight

6. $LD_{100}$ mg/kg body weight

7. $LD_{50}$ mg/kg body weight

8. $LD_{100}$ mg/kg body weight

Таблица 1

Результаты определения токсических и смертельных доз изучаемых антиокислителей

| Антиокислители (1) | Крысы (2) | | | Мыши (3) | |
|---|---|---|---|---|---|
| | минимально токсическая доза в мг на 1 кг веса (4) | $LD_{50}$ мг на 1 кг веса (5) | $LD_{100}$ мг на 1 кг веса (6) | $LD_{50}$ мг на 1 кг веса (7) | $LD_{100}$ мг на 1 кг веса (8) |
| [illegible] (BHA) | 1 000 | 2 900 | 4 500 | 1 250 | 3 000 |
| [illegible] (BHT) | 500 | 2 450 | 3 500 | 2 000 | 2 500 |
| [illegible] (PG) | 500 | 2 600 | 3 500 | 1 700 | 2 500 |

Sub-acute experiments were set up to establish functional disruption under the influence of these compounds and were given in large cumulative doses (comparative doses were dangerous to man). In observing such accumulation we fixed attention on these tests and chronic experiments in finalizing the decision of the problem of the possibility of administering phenol antioxidants in food fat. In sub-acute tests we used four groups of rats: first group received in the fat ration BHA in dose of one-fifth of the $LD_{50}$; i.e. 600 mg/kg body weight; the second group received BHT, initially one-fifth $LD_{50}$; i.e. 500 mg/kg body weight; later this dose was decreased to 250 mg/kg body weight because of poor appetite for the food at the higher BHT content; the third group received one-fifth the $LD_{50}$ of PG, 500 mg/kg body weight; the fourth group was the control group. The antioxidant was given in lard, which was mixed with the basic diet. The control group received pure lard. The lard plus antioxidant preparation necessary for setting up the experiment was prepared for us by the All Union Scientific Research Institute of the Meat Industry.

Each rat was maintained in a separate cage to better determine the quantity of feed with antioxidant action.

In observing functional states of experimental animals we relied on the following indicators.

1. Daily observation of the animal.

2. Weighing the animals once every ten days.

3. Conducting morphological studies of the blood since it is known that phenol and its derivatives often produce pathology in hematopoiesis. During the morphological studies, we counted formative elements: erythrocytes, reticulocytes, thrombocytes, leukocytes; the hemoglobin content and the leukocytic formula.

4. From this we note that the oxidative process lies at the basis of a majority of biochemical reactions of the organism and that antioxidants, possibly, in the living organisms exert influence on the processes of oxidation, we studied the activity of oxidative enzymes, especially catalase, and peroxidase. These enzymes were chosen since Tappel and Marr (1954), studied in detail the effect of propyl gallate and nordihydroquarantic acid (also a phenol antioxidant) on fourteen enzyme systems and concluded that in concentration of $1 \times 10^{-4}$ M they inhibit catalase and peroxidase activity. The inhibitory action of phenol compounds on catalase was indicated also by Goldacre and Galston (1953). Catalase was determined according to the method of Bach and Seukovova. Peroxidase was determined oxidation of indigo carmine and expressed in time (seconds), necessary for its oxidation.

5. We studied cholinesterase activity of the blood serum which, in the opinion of physiologists (K. M. Bykov, 1949; Kh. S. Koshtovants, 1946) is closely connected with the functional state of the nervous system.

6. We studied prothrombin time (which depends on the amount of prothrombin synthesized in the liver). The clinical signs of the value of this determination is the revealing of functional disturbance of the liver. All the indicators were determined for animals before the experiment and after they had been given antioxidants each two weeks. Animals were in the experiment from 68 to 82 days. At the end of the experimental period, all animals were sacrificed. Pathological examinations and histological studies of the internal organs were made. This pathological study included chemical investigation of the liver, determination of the dry weight of substances, the amount of glycogen, fats, and phospholipids

to detect possible destruction of certain aspects hydrocarbon and fat metabolism in that organ.

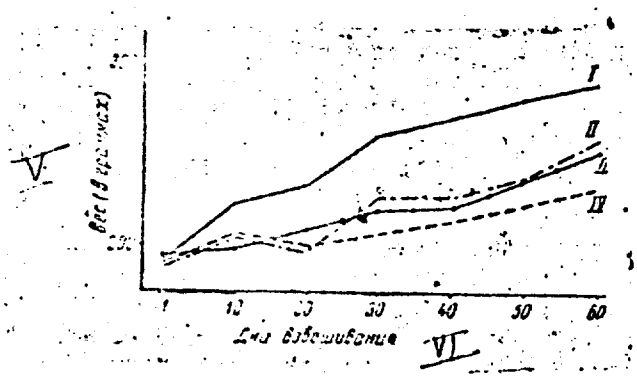
During the whole experimental period, each experimental animal was given the following quantities of one of these preparations: BHA 5--7 gms, BHT 2--4 gms, PG 4--6 gms.

Changes in behavior and external appearance of the experimental animals were not noted. The weight of the animals showed that, until administration of the antioxidants to the animals, all groups maintained their weight well. From the beginning of antioxidant administration the weight of the experimental animals began to differ significantly from the weight of the controls (figure 1). Weight lag in the animals in the experimental group was most well developed in the first month of the experiment and less well developed in the second month.

In the animals receiving BHT and BHT there was diminished blood catalase activity. In control rats toward the end of the experimental period, catalase activity was about 9.5--13.5 mg/$H_2O_2$, and in the BHA group, 6.7--8.8 mg, and in the BHT group towards the 43rd day, 6.1--8.8 mg; towards the 60th day the activity increased somewhat but did not reach catalase levels of the control animals. Propyl gallate did not produce changes in catalase activity.

FIGURE 1

CHANGE IN RAT WEIGHT DURING THE EXPERIMENTAL PERIOD



I. Weight of Control;

II. Weight of Animals Receiving PG;

III. BHA;

IV. BHT;

V. Weight in grams;

VI. Day of weighing.

FIGURE II

FAT CONTENT IN LIVER (IN DRY SUBSTANCE)

I. Control groups;

II. Animals Receiving Butyloxianisol [BHA];

III. BHT

IV. PG

V. Percent Fat

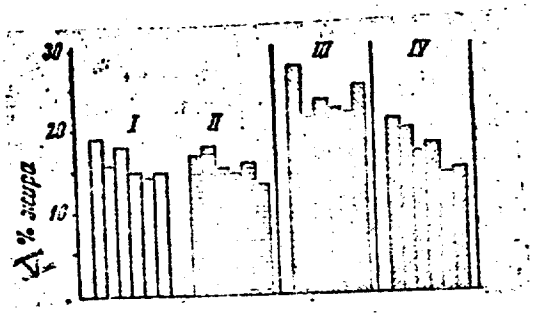



FIGURE III

CONTENT OF PHOSPHOLIPIDS IN THE LIVER
(IN DRY SUBSTANCE)

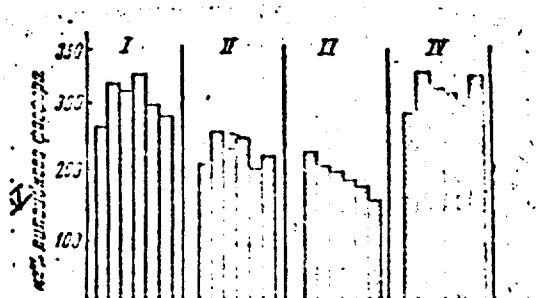


FIGURE III-- SAME CAPTIONS AS FOR FIGURE II -- EXCEPT

V. Percent Phospholipid

The blood of all three experimental groups of animals revealed a lowered peroxidase activity (the number of seconds required for oxidation of the substrate (increased). Blood peroxidase activity in control animals varied between 56--58 seconds and in the group receiving BHA by the 42nd day 105--147 toward the end of the experiment 95--135 seconds. In the BHT group, the time was 83--116 seconds towards the end of the experimental period and in the PG group, 84--110 seconds.

There was decreased cholinesterase activity in animals who had received BHT and PG.

Toward the end of the experiment the activity was as follows: In control animals 19.7--25.4% destruction of acetylcholin chloride, in the BHT group, 10.4--16.3%; in the PG group, 13.2--16.3%; in the BHA group, there was no difference from the control. Variation in morphological state of the blood and urine was not noticed. Prothrombin time in all animals did not differ from that of the controls.

There were no deep pathoanatomical changes observed. The liver weight of rats receiving BHA and BHT was higher than in the control groups and the group receiving PG. Histological examination of the internal organs, brain, lungs, stomach, small intestine, large intestine, liver, spleen, kidneys, urethra, sex organs, revealed no significant differences among the groups or difference from the control group. Only in one rat (No. 15) in the PG group was there some damage to seminal vessicles and cessation of spermatogenesis. In another rat (No. 17) of the same group, there were differences in the enlargement of lymph capillaries to the small intestine. Pathological studies were done at the Department of Food Hygiene Institute of Nutrition Academy of Medical Sciences, USSR by U. P. Kukel'.

Liver fat content was equivalent in all the groups (from 14.6--20.6%). Liver in rats receiving BHT contained somewhat more fat 20.8--27.4%) (Figure 2). In the same time livers of these animals had little lipoid phosphorous (Figure 3). This indicates that in animals receiving BHT there is some disruption of phospholipid synthesis and a yield of neutral fat in the livers as revealed by some fatty infil-

tration of liver in histological study. There was some dimunition of lipid phosphates in animals receiving BHA.

The percent content of dry substance in glycogen was approximately the same in all four groups.

The conducted acute and sub-acute experiments gave the basis for the following conclusions.

1. Phenol antioxidants BHA, BHT, and PG, were not very toxic. ($LD_{50}$ was more than 1000 mg/kg body weight and their toxicities close to one another).

2. Sub-acute tests show that phenol antioxidants, although they did not produce histological changes in the organs, were far from harmless to the animal organism:

a) experimental rats differed inweights from controls. This indicates that injected doses lead to disruption of weight gain in the experimental animals;

b) The antioxidants lowered the level of oxidative processes in the organism, particularly oxidative enzyme activity;

c) BHT and PG inhibit blood serum cholinesterate activity.

d) BHT is the most toxic. Chemical studies of the liver show that BHT exerts a harmful effect on the liver, disrupting phospholipid coupling and yielding neutral fats in the liver.

For a final conclusion on the possibility of administering phenol antioxidants in industrial practice at the present time, we will conduct chronic tests with small doses of the preparation. This is necessary to fully exclude the possible toxic action on man of fats containing BHA, BHT and Propyl Gallate.

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TRANSLATION NO. 2

# HYGIENIC EVALUATION OF NUTRIENT FATS CONTAINING PHENOL ANTIOXIDANTS

I. A. KARPLYUK

Chair of Food Hygiene [head -- professor F. Ye. Budagayn] Central Institute of Post-Graduate Medicine, Moscow

In recent years we have begun to use chemical methods of protecting fats from spoilage by adding to them small quantities (0.01--0.02%) of substances having antioxidant properties. Antioxidants inhibit oxidative processes and prolong the storage life of fat.

Judging by the literature data, the most effective antioxidants are mixture of butyloxyanisol (a mixture of two isomers of 2- and 3- tertiary butyl -4-oxianisols), butyoxytoluol (2, 6- di-tertiary butyl-4-methylphenol) and gallic acid esters (propyl, octyl, and dodecyl). In a series of countries (England, U.S.A., Sweden, Denmark, and others) these antioxidants have long been used in the industrial production of fats and fat-containing products (Tollenaar and Vos, 1956; Fincke, 1956).

In connection with the foregoing, use of butyloxyanisol, butyloxytoluol and propyl gallate for stabilization of nutrient fats in the USSR (A. K. Lebedeva, 1958; N. M. Emanuel', D. G. Knorre, U. N. Lyaskovskaya, V. I. Piul'skaya, 1955, 1958) there arose the problem of danger in the suitability of such fats for human nutrition.

Consumption of food products with strange chemical compounds is undesirable from a hygienic point of view, especially if these products are used daily in the normal course of life. Health organizations are deciding to add chemical substances to food products in cases of acute necessity only and also to remove dangers already existing from the hygienic point of view.

In series of cases, under certain conditions, the preservation of fats is impossible without the addition of antioxidants. The addition of fat of antioxidant along with other measures can practically remove physiological hazards.

In the literature, there is not enough data on the hygienic evaluation of rats containing butyloxyanisol, butyloxytoluol and propyl gallate. Therefore, we undertook experimental studies of this and to give conclusions on the possibility of the use in the food industry of the above phenol antioxidants.

The problem of the possibility of use for food of fats containing these substances was addressed as follows:

1. We studied the organoleptic properties of fats containing antioxidants, since already changes in appearance, color, odor, and taste of product can prohibit their use.

We tested the taste of cooked potatoes prepared with various types of fat containing maximum antioxidant concentrations of 0.2%. There were no observable organoleptic properties in potatoes fried in control portions of fat to which had been added butyloxyanisol, butyloxytoluol and propyl gallate.

2. In animal tests we studied toxic properties by means of establishing the lethal dose, and also determining the toxicity of these antioxidants in sub-acute tests upon the administration of relatively large doses.

This work was published by us earlier (I. A. Karplyuk, 1957, 1959).

3. The most attention in year-long chronic tests was paid to the possible toxic effect of fats containing these substances and to giving a hygienic evaluation of such fats.

Chronic t

During the first six months, rats were given 0.2% of the test fat which contained one of the antioxidants mentioned above in their daily food, on the basis of 4 mg antioxidant per kilogram of animal weight (aggravation tenfold). The control group of animals received the same quantity of pure fat.

In the second six months, the rats ate food containing 8 mg antioxidant per kilogram of weight. The ingested quantity of antioxidant was increased in the second half of the experimental period to establish which was the most toxic of the three studied compounds, since in aggravation in the first six months of the experiment we did not detect any harmful action in any of these antioxidants.

Dogs ate fat containing four percent of one of the antioxidants based on 4 mg's antioxidant per kilogram of body weight.

During the experimental period we maintained continuous observation of the total condition of the animals and their weight.

To determine possible functional changes brought about by the administration of antioxidants, animals were periodically examined (six times during the time of the experiment): morphological examination of the blood was made, the protein content of the blood serum was determined, and we studied the enzyme activity of the blood, and urine. We tested for catalase, peroxidase, and cholinesterase. Special attention was paid to the study of enzyme activity, since they are the most sensitive indicators of a substance's effect in sub-acute tests (I. A. Karplyuk, 1957, 1959).

It is suggested that the harmful action of antioxidants was not observed upon first ingestion but later. We, therefore, during five months of experiment, examined rats and determined the effect these compounds produced in subsequent generations. We noted also important physiological indicators such as motor activity, weight, quality of fur, eyes, etc. The number of secondary effects in progeny separated from the mother and maintained under experimental conditions was observed (infant mice who received 8 milograms of antioxidant per kilogram of body weight).

From the second generation (by the sixth month of the experiment) there was obtained a third generation. It also received the same basic dose of one of the three antioxidants.

Toward the end of the experimental period, we conducted morphological examinations on the blood of rats from the second and third generation, determined the enzyme activity of the blood (catalase, peroxidase, and cholinesterase), and protein content of blood serum.

Thus the possible toxic effect of phenol antioxidants was determined for three generations (104 rats) of which the first generation had been in the experiment a year and the succeeding two generations for six months.

Toward the end of the experimental period, rats were sacrificed, and also a dog by injection of an air embolism. The animals were dissected for morphological studies of their organs: cerebral hemispheres, heart, lungs, stomach, intestines, liver, spleen, pancreas, lymph nodes, kidney, urethra, sex organs, thyroid gland, adrenal gland.[1] Histological examinations of animal livers were confirmed by chemical study of the dry substances, glycogen, fat, phospholipids and cholesterol content.

All indicators agreed and biochemical studies of the blood and liver of rats were treated statistically and the reliability of different indicators determined from the animals of the experimental and control groups was calculated.

Through the whole experiment changes in rat behavior were not observed. All experimental rats differed little in weight from the control rats. The morphological picture of blood enzyme activity, serum protein content and biochemical studies of livers of rats who had received antioxidants did not differ from those in the control groups. (Tables 1 and 2).

TABLE I on following page .....

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[1]Morphological studies of the organs were conducted by an employee of the Institute of Nutrition, Academy of Medical Sciences, USSR, Yu. P. Kukel'.

# TABLE I

## RESULTS OF LIVE RAT STUDIES[2]

Результаты прижизненных исследований крыс[2]

| Группа животных (3) | Показатель (1) | | | | | |
|---|---|---|---|---|---|---|
| | (2) вес в г | | каталаза крови (в мг $H_2O_2$) | пероксидаза крови (в секундах) | холинэстераза сыворотки крови (в % разрушенного ацетилхолинхлорида) | процент белков в сыворотке крови |
| | самок (4) | самцов (5) | (6) | (7) | (8) | (9) |
| (10) Получавшая в рационе бутилкоксианизол: | | | | | | |
| (11) до опыта | 75,0±1,4 | 74,3±1,6 | 10,1±0,4 | 83,5±9,2 | 19,4±0,4 | — |
| (12) в середине опыта | 189,5±7,7 | 284,0±11,6 | 9,1±0,3 | 86,3±4,8 | 22,3±1,3 | 6,85±0,09 |
| (13) в конце опыта . . . . | 200,5±7,3 | 307,6±14,6 | 9,9±0,3 | 179,4±9,5 | 21,4±2,1 | 7,02±0,06 |
| (14) Получавшая в рационе бутилокситолуол: | | | | | | |
| (11) до опыта | 74,3±1,6 | 74,7±4,0 | 10,5±0,6 | 75,2±4,0 | 20,2±0,6 | — |
| (12) в середине опыта | 228,8±9,9 | 327,7±18,8 | 8,8±0,4 | 94,0±5,8 | 24,5±1,5 | 6,78±0,11 |
| (13) в конце опыта . . . . | 242,2±10,4 | 374,6±18,4 | 10,0±0,3 | 183,1±12,4 | 22,3±2,8 | 6,86±0,10 |
| (15) Получавшая в рационе пропилгаллат: | | | | | | |
| (11) до опыта | 72,2±3,6 | 73,5±1,5 | 8,7±0,4 | 81,3±2,3 | 20,5±0,4 | — |
| (12) в середине опыта | 194,8±4,8 | 302,7±14,1 | 8,7±0,4 | 93,3±5,1 | 26,3±1,9 | 6,95±0,09 |
| (13) в конце опыта . . . . | 210,2±7,2 | 288,0±24,9 | 9,5±0,4 | 205,0±14,8 | 20,4±2,6 | 6,85±0,16 |
| (16) Контрольная: | | | | | | |
| (11) до опыта | 72,2±3,9 | 75,2±3,8 | 9,1±0,4 | 72,7±2,9 | 19,3±0,6 | — |
| (12) в середине опыта | 2[illegible],2±8,4 | 286,2±15,8 | 8,4±0,5 | 94,4±5,7 | 21,9±1,2 | 6,82±0,10 |
| (13) в конце опыта . . . . | [illegible],2±11,4 | 300,8±10,1 | 9,6±0,3 | 203,4±11,0 | 22,4±3,3 | 7,09±0,09 |

[2] Data was obtained as M ± m, where M- average arithmetic, m- average arithmetical error.

1. Indicator
2. Weight in grams
3. Group of Animals
4. Females
5. Males
6. Blood Catalase (in Mg $H_2O_2$)
7. Blood Peroxidase (in seconds)
8. Serum cholinesterase (in % of destroyed acetylchloride)
9. % protein in blood serum
10. Butyloxyanisol obtained from ration
11. Before the experiment
12. In the middle of the experiment
13. At the end of the experiment
14. Butyloxytoluol received in ration
15. Propyl gallate received in ration
16. Control

The peroxidase activity at the 4th month and cholinesterase at the eighth month in animals who received propyl gallate was higher than in the control group. In two animals there was a loss of liver weight, (weighed less than five grams), in three a decreased glycogen and phospholipid content.

TABLE II on following page .....

# TABLE II

## RESULTS OF BIOCHEMICAL STUDIES OF RAT LIVER[1]

Результаты биохимических исследований печени крыс[1]

| Показатель (1) | Подопытные группы, получавшие в рационе (2) | | | Контрольная группа (6) |
|---|---|---|---|---|
| | бутилоксианизол (3) | бутилокситолуол (4) | пропилгаллат (5) | |
| (7) Вес печени (в г) | 8,68±0,53 | 9,09±0,95 | 8,97±0,80 | 8,76±0,42 |
| (8) Сухой остаток (в %) | 28,6±0,37 | 28,6±0,49 | 27,3±0,68 | 28,9±0,43 |
| (9) Гликоген (в г %) | 1,70±0,26 | 1,84±0,21 | 1,99±0,29 | 1,94±0,24 |
| (10) Жир (в % на сухое вещество) | 17,3±0,38 | 18,5±0,57 | 17,9±0,75 | 17,5±0,59 |
| (11) Липоидный фосфор (в мг% на сухое вещество) | 265,0±12,1 | 248,0±13,5 | 218,0±19,3 | 268,0±16,5 |
| (12) Холестерин (в г% на сухое вещество) | 1,32±0,06 | 1,39±0,08 | 1,15±0,03 | 1,74±0,13 |

1. Indicator
2. Experimental group receiving in ration:
3. butyloxianisol
4. butyloxitoluol
5. propyl gallate
6. Control Group
7. Liver weight in grams
8. dry residue in %
9. Glycogen in gram %
10. Fat in % of dry weight
11. Phospholipid in milligram % of dry weight
12. Cholesterol in gram % of dry weight

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[1] Data expressed as M ± m, where M is the average arithmetic, and m the error of the average arithmetic.

The pathoanatomical and histological studies confirmed data on the safety of the compounds studied with the exception of propyl gallate. In three males that received propyl gallate, degenerative changes in the seminal vesicles occurred. The addition to rat rations of fat with butylantioxidants did not interfere with the development of two generations of rats. In the majority of published studies on rats of second and third generation changes were noted only in animals that had received propyl gallate; blood catalase activity was increased.

A round of biochemical studies in experiments on dogs was not as decisive. In these, as in other larger animals, it is possible to conduct such studies which cannot be conducted on rats because of the impossibility of obtaining sufficient quantities of study material. Beside the above noted biochemical criteria, we determined the residual nitrogen content, blood cholesterol levels, and made Wrightman coagulation tests and Takata flocculation tests to determine the possibility of changing liver function and other indices of the metabolism of substances in the body.

Dog experiments showed no toxic effect for these three compounds.

Morphological studies of the blood showed that the number of formative elements in experimental and control dogs varied within one or another normal limits.

The blood enzymes studied (catalase, peroxidase, cholinesterase) did not change in dogs that had fed on butyloxianisol, butyloxitoluol, or propyl gallate.

In determining the protein and cholesterol content in the blood serum, no destruction of synthesis of these substances by the liver was measurable.

In studying residual blood nitrogen, it was determined that the control group figures did not exceed 44 milogram % variation from the experimental group.

The Takata reaction did not reveal qualitative changes in blood protein fractions. Precipitation and blood clotting after the addition of 0.5% mercury choloride did not differ between the experimental and control group.

The Volkman coagulation test did not indicate changes in electrolyte level of the blood of experimental animals. Both experimental and control animals had about

the same coagulation level. In connection with this, one can consider that coagulation did not differ appreciably from that of normal dogs.

In Table III results of the biochemical investigation of the livers of experimental and control animals are presented.

TABLE III

RESULTS OF BIOCHEMICAL STUDIES OF DOG LIVERS

Результаты биохимических исследований печени собак

| Показатель (1) | Подопытные животные, получавшие в рационе (2) | | | | | | Контрольные животные (6) | |
|---|---|---|---|---|---|---|---|---|
| | бутилоксианизол (3) | | бутилокситолуол (4) | | пропилгаллат (5) | | | |
| | Мямлик | Дамка | Ла | Шустрик | Мух | Джильда | Лайка | Белка |
| (7) Сухой остаток (в %) | 26,7 | 27,6 | 28,7 | 28,0 | 29,0 | 28,0 | 29,3 | 29,3 |
| (8) Гликоген (в г%) | 0,84 | 1,42 | 2,44 | 1,74 | 2,0 | 1,16 | 2,19 | 1,97 |
| (9) Жир (в % на сухое вещество) | 19,1 | 17,4 | 17,8 | 17,1 | 18,5 | 16,8 | 19,0 | 18,7 |
| (10) Липоидный фосфор (в мг% на сухое вещество) | 245,0 | 168,0 | 292,5 | 268,7 | 266,0 | 295,0 | 285,0 | 256,0 |
| (11) Холестерин (в г% на сухое вещество) | 1,76 | 1,70 | 1,56 | 1,90 | 1,62 | 1,76 | 1,47 | 1,75 |

1. Indicator
2. Experimental animals receiving in ration:
3. butyloxianisol
4. butyloxitoluol
5. propyl gallate
6. Control Group
7. Dry residue in %
8. Glycogen in %

9. Fat in % of dry weight

10. Phospholipid in milogram % of dry weight

11. Cholesterol in gram % of dry weight

As is evident from Table III the indicators for experimental animals do not differ from those of control dogs. Only in the dog Myanlik (in the butyloxianisol group) the liver glycogen level was somewhat lower than in the other animals. We consider it necessary to note that, these dogs were not sacrificed quickly and therefore there is a reason for diminution in glycogen quantity.

Histological studies of the central nervous system and internal organs did not reveal changes which could be attributed to the introduction into the organism of these phenol antioxidants. Thus, on the basis of determining the toxicity of the three phenol antioxidants in year-long chronic tests, one can conclude that butyloxianisol and butyloxitoluol in quantities recommended for use in nutrient fats are not toxic.

This experiment brought us to the conclusion that butyloxianisol and butyloxitoluol in 0.01-0.02 percent level in fats can restrain oxidative processes without endangering their hygienic position.

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# THE EFFECT OF ANTIOXIDANTS OF FOOD FATS (BHA AND BHT) ON CERTAIN ASPECTS OF FAT METABOLISM IN ANIMALS

I. A. KARPLYUK

Department of Food Hygiene [Head - Professor Ye. Budagyan] Central Institute of Post Graduate Medicine, Moscow

The protection of fats and fat-containing products from oxidation is of great economic significance and is also a hygienic problem. Fat oxidation and polymerization products exert a harmful action on the organism (toxic, carcinogenic, or pre-carcinogenic). Therefore, attention is turned to the physical and chemical methods of protecting fats from oxidation.

Since fat oxidation is of a chain reaction-type character, the reaction can be slowed significantly with the aid of an oxidation inhibitor. Of the synthetic antioxidants, the most effective and useful in the USSR are butyloxianisol [BHA], butyloxitoluol [BHT], esters of gallic acid.

Since antioxidants are fed in chemical substances to the organism, their use in the food industries should be decided upon only if they are absolutely safe oxidation inhibitors which do not change the organoleptic properties of the product.

Basic work carried out in preceding years at the Chair of Food Hygiene of the Central Institute of Post-Graduate Medicine [1--3], BHA and BHT were shown to be comparatively low toxicity substances. Low quantities (0.001--0.02% in relation to fat ration in most of the conducted studies was judged harmless to the body.

However, the problem of studying possible toxic effect of synthetic antioxidants in the organism remains. There is still a series of interesting but until now insufficiently studied problems in the field of fat metabolism in the realm of metabolism of fats containing antioxidants.

In the literature we have signs indicating that antioxidants affect fat metabolism. In the experiments of Johnson and co-authors [4], rats ingesting BHA and BHT in quantities of 0.1--0.5% of the diet had increased fat stability.

Johnson and Hewgill [5] showed that feeding BHA and especially BHT to animals in concentrations of about 0.3--0.5% led to increased contents of cholesterol in the blood serum. Small BHT doses (0.001%) did not produce such changes. Day and co-authors [6], showed the positive inter-relation between BHT metabolism (especially large doses), fatty acids and cholesterol in phospholipid transport. These scientists conducted experiments with rather large doses of antioxidants. (Aggravation to 100--500 times). Results of the work showed effect on fat metabolism of thiol antioxidants.

We undertook to follow the effect of feeding of fats containing BHA and BHT on certain aspects of fat metabolism.

Experiments were made on male rats with an initial weight between 120 and 150 grams, who were given, during thirty to thirty-five days, the following rations: 15 grams of feed concentrate (containing wheat meal, stabilizers, bone meal, minerals, fish meal, blood meal, powdered eggs, dry milk, hydrolyzed yeast, and molasses), 10 ml of whole milk, 3 grams of oil and 1 gram of lard containing 0.006% iodine. This ration contained 20% protein, 25% fat, 55% carbohydrate. The animals in the control group received pure fat without additions; the animals in the first group were given pure fat with BHA, the animals in the second group the same fat with BHT. The antioxidant was added in four mg/Kg (aggravation ten times). Both control and experimental groups consisted of ten animals apiece.

The animals were studied by the following tests: weight of rats, cholesterol and phosphorous lipid content in the blood serum, number of ketone bodies in the blood. Since the role of the liver in fat metabolism is very great,

we made detailed studies of that organ. We determined its weight, the relative weight of the liver to body weight, content of dry substance, glycogen, fat, phosphor lipid in the liver, iodine number of the fat in the liver.

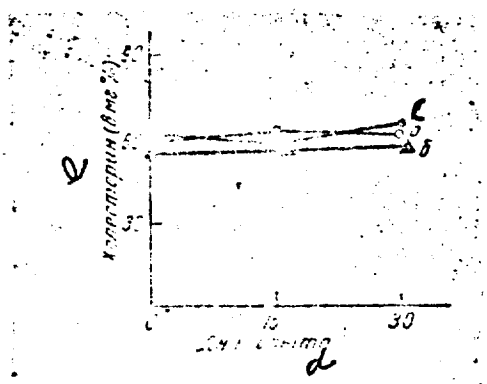
Cholesterol in the blood serum was determined by a colorimetric method worked out by M. A. Levchenko [7]. Phospholipid in blood serum in the liver was studied by Brewer's method [8]. To determine ketone bodies we used a differation method worked out by Soxlet. We extracted liver with petroleum ether, and determined the iodine number of the fat. Glycogen in the liver was determined by Fluger's method with modifications worked out by A. P. Belova [10].

The obtained results were treated by the variation statistical method. As a result of the studies carried out, it is possible to note the following:

The addition of fat of BHA and BHT does not affect food intake and animal weight.

Acetone, b-oxyoleic and aceto-acetic acids were normal products of the metabolism of higher fatty acids. Normally, they enter through the acetylation co-enzyme A and the TCA cycle to carbon dioxide and water and their content in the blood and water is very small.

Figure 1 ----- Cholesterol content in the blood serum in experimental and control animals (average data for group).



a. Animals receiving fat with BHA;

b. Animals receiving fat with BHT;

c. Control group receiving pure fat;

d. Days of the experiment;

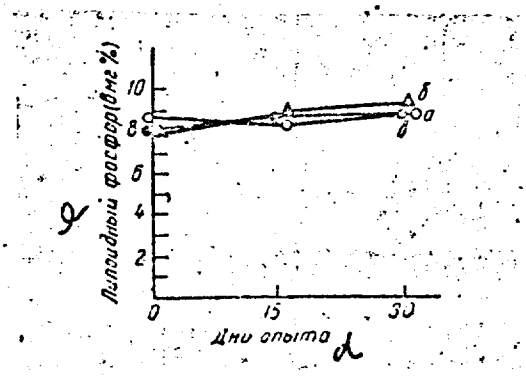
e. Cholesterol in milligrams %.

FIGURE 2

PHOSPHO-LIPID CONTENT IN BLOOD SERUM OF EXPERIMENTAL AND CONTROL ANIMALS (AVERAGE DATA FOR THE GROUP)

Captions the same as for Figure 1, except for E:

e. Phospho-lipids in milligrams %.

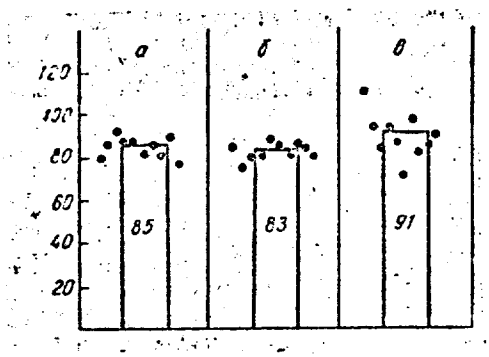



In the decrease of oxidative processes in the organism, a possible consequence of the administration to the organism, of sufficiently active oxidation inhibitor, is that the ketone bodies in the blood can rise. Hypo-ketonemia serves as an indicator of insufficient higher fatty acid oxidation in the liver, as observed in the diseased liver. During the experimental period, three days of study of the content of the ketone bodies in rat blood was made and we did not note any increase or decrease in their number in the blood ($P. > 0.05$). One can conclude

that the administration to the animals of antioxidants in comparatively low doses does not affect the formation of or oxidation of ketone bodies.

FIGURE 3

IODINE NUMBER OF LIVER FAT IN EXPERIMENTAL AND CONTROL ANIMALS




a. Animals receiving fat with BHA;

b. Animals receiving fat with BHT;

c. Control animals receiving pure fat;

Dark circles -- Iodine number of liver fats in the individual animals;

Columns with numbers indicate the average arithmetical number (N) in each group.

The cholesterol and phospholipid levels are shown in Figures 1 and 2.

We, in our preceeding studies, on the feeding of dogs with BHA and BHT also did not observe any increase in blood cholesterol levels. [1] Thus, our experiments do not confirm the effect these antioxidants and cholesterol and phospho-lipids have in the blood serum. BHA and BHT can be given in doses ten times that in the test.

The table presents indicators characterizing the state of the liver in animals receiving fat with BHA and BHT, and in the controls.

TABLE

RESULTS OF LIVER STUDIES IN EXPERIMENTAL AND CONTROL ANIMALS

Результаты исследования печени подопытных и контрольных животных

| Показатель (1) | Группы животных (2) | | | | |
|---|---|---|---|---|---|
| | контрольная (3) | 1-я (получали бутил-оксианизол) (4) | | 2-я (получали бутилгидрокситолуол) (5) | |
| | $M_к \pm m_{M_к}$ | $M_1 \pm m_{M_1}$ | $P$ | $P_2 \pm m_{M_2}$ | $P$ |
| (6) Вес печени (в г) | 9,18±0,32 | 9,01±0,41 | >0,5 | 7,99±0,11 | <0,05 |
| (7) Отношение веса печени к весу тела (в %) | 3,5±0,2 | 3,3±0,15 | >0,25 | 3,2±0,1 | <0,1 |
| (8) Содержание сухих веществ (в %) | 29,9±0,3 | 29,7±0,3 | >0,5 | 29,4±0,3 | <0,25 |
| (9) Гликоген (в г%) | | | | | |
| (10) Липоидный фосфор (в мг% на сухое вещество) | 1,83±0,22 | 1,71±0,22 | >0,5 | 1,72±0,13 | >0,5 |
| (11) Жир (в г% на сухое вещество) | 216±7 | 225±9 | >0,5 | 218±10 | >0,25 |
| | 17,8±0,6 | 17,3±0,6 | 0,5 | 18,0±0,3 | >0,5 |

1. Indicator
2. Animal group
3. Control
4. BHA group
5. BHT group
6. Liver weight in grams
7. Relative weight of liver to body weight
8. Content of dry substances in %
9. Glycogen in grams %
10. Phospho-lipids in mg% of dry weight
11. Fat in grams % of dry weight

In animals receiving fats with BHT, liver weight decreased. However, a comparison of the relative liver weight to body weight was a more reliable characteristic of changes in the dimensions of the organism is evident in that the statistical reliability of the difference between the experiments and controls is nil. In a given case it is possible to speak only of a certain tendency to diminuition of this relationship in experimental animals.

The dry substance content, glycogen, phospholipids, and liver fat, did not differ between control and experimental animals.

The iodine number of fat in the liver, which indicates its degree of unsaturation, was lower in animals who had received fats with BHT ($P = 0.05$). This agrees with Johnson's data [4], also the noted diminuition of the iodine number of fat in the liver holds in the feeding of BHT in concentrations of 0.1 and 0.5%. In animals who had received BHA in their ration, there was no statistically reliable difference. (P greater than 0.1).

One can believe that the diminuition in iodine number is connected with anti-oxidants inhibiting the oxidation processes of fatty acids in the liver. Possibly, dehydration of fatty acids in which saturated acetyl co-enzyme A leads changes to $alpha_1$ beta unsaturated co-enzyme A.

## CONCLUSIONS

1. 30--35 days of feeding BHA did not affect fat metabolism in the animals studied.

2. Feeding of fats with BHT led to a lowering of the iodine number of liver fats in experimental animals, which indicates that BHT somehow affects the oxidation of fatty acids in the organism.

3. From the point of view of choosing the most effective and safest food antioxidant, more studies are needed on the possible toxic effect of phenol antioxidants on the organism.

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# Effect of Antioxidants on Life-Span of C57BL Mice[1]

Robert R. Kohn, PhD, MD[2]

In a study based on the free radical theory of aging, Harman (1968) determined the effects of antioxidants on life-span of the long-lived $LAF_1$ mouse strain. Two of the free radical inhibitors tested by Harman increased the 50% survival times, but none of the agents caused an increase in maximum life-span.

The experiments to be reported here were undertaken in attempts to confirm or extend Harman's findings, using the hardy and long-lived C57BL/6J mouse strain. The two agents found effective by Harman were used; 2-mercaptoethylamine hydrochloride (MEA) and 2,6-di-tert-butyl-4-methylphenol (butylated hydroxytoluene-BHT).

In designing these studies, it was decided that controls would be valid only if their mortality data were optimal for the C57BL/6J strain. The longest life-spans which appear to have been reported for this strain were obtained by The Jackson Laboratory for the Pedigreed Expansion Stocks (Russell, 1966). These mortality data were used for evaluating control data.

Since mean life-span can vary considerably in response to environmental and nutritional factors, an agent would be considered to have inhibited aging processes only if it lengthened maximum life-span beyond that of the most long-lived controls. Also, since it has been shown that underfeeding and slowing of growth result in lengthened life-span (McCay, 1952), effects of antioxidants on body weight were determined.

## Materials and Methods

Weanling females and retired breeder mice of the C57BL/6J strain were obtained from The Jackson Laboratories, Bar Harbor, Maine. For each experiment, experimental and control groups were from the same shipment. Animals were housed, 20 to a cage, in 15″ X 13″ X 6¾″ high polycarbonate cages with stainless steel wire bar lids with filter caps. Bedding was hardwood chips and aspen shavings. Distilled drinking water and Wayne Mouse Breeder Blox were given *ad libitum*. This diet contains the antioxidant alpha-tocopherol at a concentration of 35.2 ppm. Diet containing added antioxidants at required concentrations was prepared monthly. Lights in the animal room were on 14 hours and off 10 hours. Attempts were made to maintain room temperature at 72 F., but occasional air conditioning failures caused temperatures to rise to over 80 F. for periods of 12 to 15 hours.

Three experiments were carried out: 1) BHT and MEA, each administered to groups of 100 retired breeders, with 60 controls, 2) MEA given to 50 retired breeders, with 50 controls, and 3) BHT given to 100 female weanling mice, with 100 controls. The experiments overlapped in time and were carried out with three separate shipments of mice.

Animals were observed daily, and each death was recorded. Mice were weighed each week.

## Results

The MEA dosage was kept at 1% in both experiments (Figs. 1 & 2). BHT in high doses caused early weight losses and deaths, necessitating adjustments in quantities administered (Figs. 1 & 3). Animals receiving antioxidants did not attain control weights (Figs. 1, 2, & 3).

The best mortality data for control mice were a 50% survival time of 121 weeks and a maximum life-span of 148 weeks (Fig. 1). These compare with Jackson Laboratory data of 100 and 128 weeks, respectively, and are

[1] Supported by grants from the National Institute of Child Health and Human Development, NIH.
[2] Institute of Pathology, Case Western Reserve University, Cleveland 44106.

considered to represent optimal life-spans for the mouse. In the experiment in which control mice had these life-spans, MEA and BHT had no effect on either 50% survival time or maximum life-span (Fig. 1).

In one experiment, controls had a 50% survival time of 113 weeks, but a maximum life-span of only 125 weeks (Fig. 2). In this experiment, MEA had no effect on the 50% survival time, but caused a lengthening of maximum life-span to 147 weeks; maximum life-span was similar to that of controls shown in Figure 1.

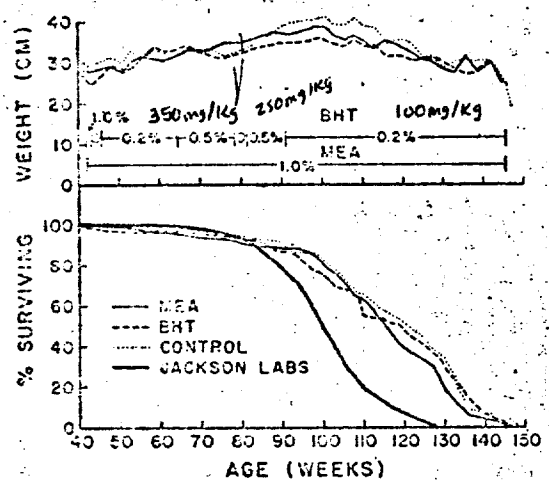


Fig. 1. Weights and survival of retired breeder C57BL mice treated with MEA and BHT. Dosages as percentage of diet are indicated. Survival data of Jackson Laboratories are included.

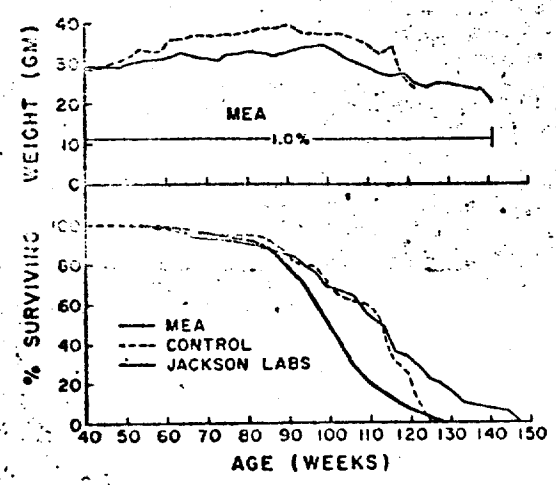


Fig. 2. Weights and survival of retired breeder C57BL mice treated with MEA. MEA dosage, as percentage of diet is indicated. Survival data of Jackson Laboratories are included.

In the third experiment, controls showed a 50% survival time of only 90 weeks, but a maximum life-span of 146 weeks, largely due to survival of one mouse for 15 weeks after the others had died (Fig. 3). BHT extended the 50% survival time to 113 weeks, and maximum life-span to 151 weeks. The 50% survival times would have been significantly longer if early deaths in the BHT-treated mice and controls were omitted. The effect of BHT in this experiment was to cause 50% survival time and maximum life-span to attain optimal control values, as shown in Figure 1.

## Discussion

The findings in these experiments are similar to those of Harman (1968). When survival of control mice is optimal, these antioxidants are without effect on life-span. When survival of controls is suboptimal, as manifested by shortening of either 50% survival time or maximum life-span, BHT and MEA cause lengthening of these life-spans. In no case, however, do the antioxidants increase the 50% survival time or maximum life-span significantly beyond values obtained from control mice surviving under optimal conditions.

It is concluded that antioxidants evaluated in this study do not inhibit those processes which determine maximum life-span, but that they do inhibit some harmful environmental or nutritional factors which cause control mice to show suboptimal survival data in some experiments. A likely possibility is that these agents prevent the oxidation of essential nutrients in the diet.

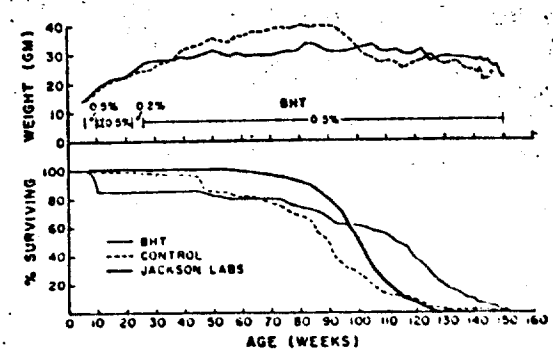


Fig. 3. Weights and survival of female C57BL mice treated with BHT. BHT dosage, as percentage of diet is indicated. Survival data of Jackson Laboratories are included.

## Summary

Effects of the antioxidants 2-mercaptoethylamine hydrochloride and butylated hydroxytoluene on mouse life-span were determined. When control mice had maximum 50% survival times and life-spans, the agents were without effect. When control survival was suboptimal, the antioxidants increased life-spans, but not beyond optimal control values. It was concluded that antioxidants do not inhibit aging, but inhibit some harmful environmental or nutritional factor.


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# The excretion of [$^{14}$C] butylated hydroxytoluene in the rat

L. G. LADOMERY, A. J. RYAN AND the late S. E. WRIGHT

The excretion of small doses of [$^{14}$C]labelled butylated hydroxytoluene has been examined in the rat. Parenteral doses are excreted slowly in urine and faeces over 4 days. Biliary excretion is rapid. The biliary radioactivity is absorbed readily from the gut and a rapid enterohepatic cycle has been found to operate over at least 96 hr.

BUTYLATED hydroxytoluene (BHT, 3,5-di-t-butyl-4-hydroxytoluene) is an antioxidant used widely in foods. It is therefore important that the toxicology, distribution, metabolism and excretion of this compound should be well understood. Dacre (1961) and Akagi & Aoki (1962) studied its metabolism in rabbits. The quantitative urinary excretion of isotopically labelled BHT has been examined by several workers. Golder, Ryan & Wright (1962) recovered only 33% of the radioactivity in urine from rats given small doses of tritiated BHT. This result was confirmed by Ladomery, Ryan & Wright (1963) using [$^{14}$C]BHT. Daniel & Gage (1965), using higher doses of [$^{14}$C]BHT found similar urinary excretion and also noted high faecal excretion. All these authors found a slow rate of excretion, which was not complete even after a week.

The present paper is concerned with the biliary excretion by rats of small doses of [$^{14}$C]BHT.

## Experimental

[$^{14}$C]3,5-Di-t-butyl-4-hydroxytoluene (2,6-di-t-butyl-*p*-cresol; [$^{14}$C]BHT) was purchased from New England Nuclear Corp., Boston, Mass., U.S.A. It was used as 0·02% solution in 50% aqueous ethanol.

### METABOLIC EXPERIMENTS

White male rats of 300–350 g were given [$^{14}$C]BHT (100 $\mu$g, except where otherwise stated) in aqueous ethanol (0·5 ml) either by intraperitoneal or intravenous injection. Urine and faeces were collected in a metabolic cage fitted with an all-glass separating device. Bile was collected from cannulated rats maintained under urethane anaesthesia. Ringer solution (1 ml) was given every 3 hr to replace lost water and the animals were kept warm. The "linked animal experiment" was carried out by passing the bile cannula from one rat into the duodenum of a second rat. This cannula was cut and joined with a short section of a hypodermic needle in order to check bile flow. The first rat was raised 5 cm above the second to assist the flow of bile into the duodenum. Bile was collected from the second rat.

### RADIOACTIVE COUNTING

Urine and bile were counted directly by the addition of 0·1–0·2 ml to 10 ml of scintillation solvent consisting of 2,5-diphenyloxazole (5·0 g), 1,4-bis-2-(5-phenyloxazolyl)benzene (0·05 g), absolute ethanol (300 ml)

From the Department of Pharmacy, University of Sydney, N.S.W., Australia.

and toluene (300 ml). Faeces were dried, weighed and a powdered aliquot extracted continuously with methanol. Aliquots of the methanolic solution were counted as above. Tissues were homogenized in ethanol, centrifuged and the supernatant assayed. The contents from the small intestine were diluted with methanol, centrifuged and the supernatant counted. The contents of the large intestine were treated in the same way as the faeces. The samples were counted in a Packard Scintillation Counter, model 3314. Counting efficiencies were determined with an external source.

## Results

The low urinary excretion of [$^{14}$C]BHT observed earlier (Ladomery & others, 1963) led us to examine the faeces of dosed animals for radioactivity. Table 1 shows that over 4 days 37% of a parenteral dose of

TABLE 1. DAILY URINARY AND FAECAL EXCRETION OF RADIOACTIVITY AFTER A SINGLE INTRAPERITONEAL DOSE OF [$^{14}$C]BHT

| Day | % Excreted in urine* | % Excreted in faeces* |
|---|---|---|
| 1 | 5·7 ± 0·5 | 15·7 ± 1·0 |
| 2 | 10·5 ± 0·8 | 7·5 ± 0·3 |
| 3 | 10·3 ± 0·6 | 7·3 ± 0·1 |
| 4 | 5·5 ± 0·4 | 5·4 ± 0·4 |
| Total | 32·0 ± 0·6 | 36·9 ± 1·2 |

* ± Standard deviation; 6 rats used.

[$^{14}$C]BHT was excreted by this route. The urinary excretion agreed with our previous results. Since only about 70% of the dose was accounted for, the general distribution of radioactivity from [$^{14}$C]BHT in rats was examined. The results are in Table 2. Four groups of six rats were

TABLE 2. DISTRIBUTION OF RADIOACTIVITY IN RATS AFTER A SINGLE INTRAPERITONEAL DOSE OF [$^{14}$C]BHT. Values are the percentage of the administered dose; urine and faeces are cumulative

| Day | Urine | Faeces | Contents of small intestine | Contents of large intestine | Liver | Spleen | Kidney | Intestinal wall | Total accounted for |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 4·5 | 7·6 | 15·9 | 6·1 | 2·1 | 0·03 | 0·03 | 7·5 | 44·8 |
| 2 | 15·5 | 20·9 | 9·8 | 4·7 | 1·8 | 0·02 | 0·03 | 4·5 | 57·8 |
| 3 | 25·5 | 29·1 | 9·6 | 3·9 | 1·0 | 0·02 | 0·03 | 2·9 | 69·8 |
| 4 | 31·2 | 37·9 | 7·4 | 2·1 | 0·9 | 0·13 | 0·13 | 1·6 | 81·3 |

dosed. Urine and faeces were pooled at 24-hourly intervals. At the same time a group of rats was killed and some of the tissues examined for radioactivity. The urinary and faecal excretion agree well with those already found. Little activity appeared in the liver, kidney or spleen. However, significant amounts appeared in the intestinal contents and the intestinal wall. This totalled about a third of the radioactivity remaining in the body.

From these results it seemed likely that there was some biliary excretion of radioactivity from [$^{14}$C]BHT and possibly an enterohepatic cycle. The

biliary excretion of [$^{14}$C]BHT was examined and found to be surprisingly high. After 6 hr, radioactivity equivalent to 95% of an intravenous dose and 52% of an intraperitoneal dose appeared in the bile (Table 3).

TABLE 3. BILIARY EXCRETION OF RADIOACTIVITY BY RATS AFTER SINGLE DOSES OF [$^{14}$C]BHT

| Time (hr) | % Excreted from 100 μg given intravenously* | % Excreted from 100 mg given intraperitoneally* | % Excreted from 10 mg given intravenously* |
|---|---|---|---|
| 1 | 46·6 ± 7·5 | 16·0 ± 4·5 | 28·5 ± 7·8 |
| 2 | 23·0 ± 8·0 | 15·5 ± 4·6 | 28·9 ± 6·5 |
| 3 | 12·6 ± 2·6 | 8·9 ± 1·1 | 8·2 ± 2·0 |
| 4 | 4·7 ± 1·8 | 5·2 ± 1·3 | 3·3 ± 0·5 |
| 5 | 2·9 ± 0·6 | 3·4 ± 0·7 | 1·5 ± 0·4 |
| 6 | 1·9 ± 0·4 | 2·8 ± 0·2 | 1·0 ± 0·2 |
| Total | 93·7 ± 11·6 | 51·8 ± 6·6 | 71·4 ± 10·4 |

* ± Standard deviation; 6 animals used in each experiment.

These high recoveries supported the idea of an enterohepatic cycle of BHT metabolites. This cycling was confirmed using pairs of rats with the bile duct cannulated, in which the cannula from one rat (A) was inserted into the duodenum of the second (B). Bile was collected from rat B after rat A was given an intravenous dose of [$^{14}$C]BHT. Nearly 30% of the dose given to rat A was excreted by rat B after 10 hr (Table 4).

The efficiency of the biliary excretion of BHT was shown when 10 mg was given intravenously. In this instance over 70% of the dose was recovered in the bile after 6 hr (Table 3).

TABLE 4. BILIARY EXCRETION OF RADIOACTIVITY BY LINKED RATS AFTER A SINGLE DOSE OF [$^{14}$C]BHT

| Time (hr) | % dose excreted* | % dose excreted per hr |
|---|---|---|
| 2 | 0·2 ± 0·07 | 0·10 |
| 4 | 5·1 ± 1·4 | 2·53 |
| 6 | 7·6 ± 0·6 | 3·80 |
| 8 | 8·0 ± 1·8 | 4·01 |
| 10 | 9·0 ± 1·3 | 4·50 |
| Total | 29·9 ± 2·7 | |

* ± Standard deviation; 5 pairs of rats used.

With this demonstration of the enterohepatic cycling of BHT metabolites it became important to determine for how long the cycle functioned. Sixteen rats were given parenteral doses of [$^{14}$C]BHT and in four of them the bile duct was cannulated every 24 hr. Radioactivity appeared in significant amounts in the bile even 96 hr after dosing, when nearly 10% of the dose was excreted (Table 5).

The efficiency of the biliary excretion was demonstrated in an additional experiment. Bile obtained from a rat given [$^{14}$C]BHT was injected intravenously (1 ml) into rats. Bile collected from these animals contained 93·0 ± 2·0% of the injected radioactivity after 6 hr. None was found in the bladder contents. This emphasizes the remarkable affinity of the liver for BHT derivatives. The bile contains mainly conjugates (Ladomery, Ryan & Wright, 1967) and it might be expected

TABLE 5. THE RATE OF BILIARY EXCRETION OF RADIOACTIVITY AT 24-HOURLY INTERVALS BY RATS AFTER A SINGLE INTRAPERITONEAL DOSE OF [$^{14}$C]BHT

| Time after dosing (hr) | % dose excreted in 6 hr* | % dose excreted/hr* | Predicted % dose excreted/hr | Rate of excretion as % dose remaining/hr |
|---|---|---|---|---|
| 24 | 23·0 ± 4·1 | 4·3 ± 0·7 | 3·9 | 4·8 |
| 48 | 20·5 ± 3·0 | 3·4 ± 0·5 | 2·9 | 5·3 |
| 72 | 13·3 ± 2·0 | 2·3 ± 0·3 | 2·2 | 4·8 |
| 96 | 9·6 ± 1·6 | 1·6 ± 0·3 | 1·4 | 5·1 |
| | | | | mean 5·0 ± 0·2 |

* ± Standard deviation; 4 groups of 4 rats used.

that intravenous injection would lead to more urinary excretion than was found. However, Williams, Millburn & Smith (1965) have found high biliary excretion of certain glucuronides. It is clear that there is no simple relation between polarity and excretion route.

## Discussion

The present work has confirmed the low urinary excretion of tritiated BHT found by Golder & others (1962). The slow urinary excretion of [$^{14}$C]BHT led us to examine the faecal excretion of activity. After a single parenteral dose urinary excretion is about 32–35% and faecal excretion is 35–37%, accounting for 70% of the radioactivity after 4 days (Table 2).

The result suggested that there might be some accumulation of BHT in tissues. However, under our dosage conditions little radioactivity was found in the organs examined. The liver with 2% contained most. Examination of the intestinal contents, however, revealed that these, together with the gut wall, contained about 30% of the activity remaining in the body after allowing for the urinary and faecal excretion (Table 3). Tye, Engel & Raplen (1965) found only a small amount of BHT in the tissues at much higher dose levels (44 mg/kg) than used in the present study. Daniel & Gage (1965) accounted for about 80% of oral doses of BHT. Faecal excretion in their experiments was much higher than that found in this work. However, this may be due to poor absorption of the BHT since it was given in olive oil, in which it is readily soluble. The laxative properties of olive oil could also be a factor in these results. The slow excretion and the constancy of the radioactive pool in the gut suggested that biliary excretion of BHT might be important. This was shown to be the case when 95% of an intravenous dose of [$^{14}$C]BHT appeared in the bile of cannulated rats (Table 3). An intraperitoneal dose of [$^{14}$C]BHT was excreted to the extent of 50% in the bile. This slower rate of excretion of a parenteral dose may be due to the rate of absorption from the peritoneal cavity. However, in both instances the rate of biliary excretion of radioactivity is greater than the daily urinary or faecal excretion. Coupled with the remarkable constancy of the proportion of radioactivity in the gut it is clear that enterohepatic cycling takes place.

Proof of this was obtained from the "linked animal experiment" (Table 4). The 30% of radioactivity recovered in the bile of rat B indicates that the circulation between bile and intestines is rapid. The

steady state attained after about 8 hr (Table 4) could be due to an active intestinal absorption process which becomes saturated. This is supported by the fact that intravenous injections of [$^{14}$C]BHT at 100 times our usual dose level are excreted to the extent of 71% of the dose in bile (Table 3) showing that the biliary system is freely permeable to the BHT metabolites. From Table 4 the steady state excretion of BHT metabolites in the bile approaches 5% of the residual radioactivity per hr. Using this figure, the biliary excretion of radioactivity was calculated for various time intervals after a parenteral dose. Table 5 shows the results obtained with rats given [$^{14}$C]BHT and cannulated at the bile duct 24–96 hr after dosing compared with the predicted excretion. A plot of the logarithm of the per cent dose/hr excreted (Wagner, 1961) against mean collection time for the experimental and predicted figures in Table 5 gives two straight and parallel lines. This provides further support that the enterohepatic circulation of BHT metabolites follows first order kinetics at the rate of approximately 5% of the remaining radioactivity per hr.

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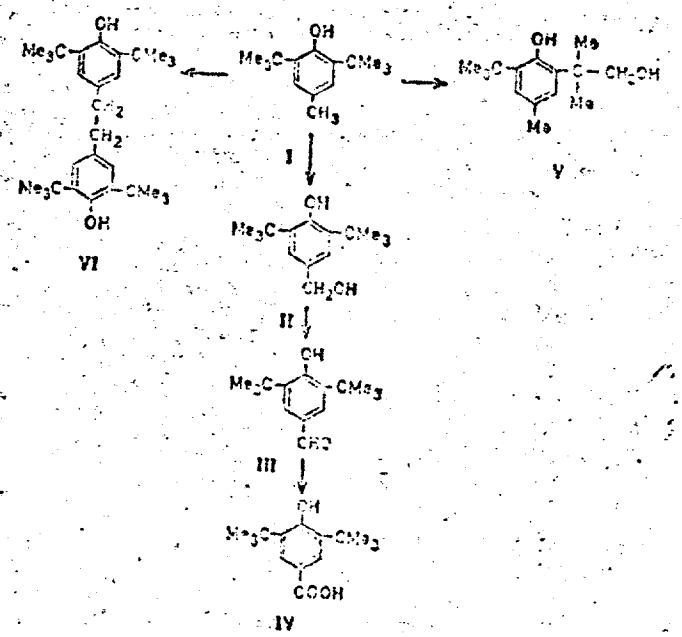
# The biliary metabolites of butylated hydroxytoluene in the rat

L. G. LADOMERY, A. J. RYAN AND the late S. E. WRIGHT

The biliary metabolites from intravenous and intraperitoneal doses of small amounts of [$^{14}$C]butylated hydroxytoluene have been separated and estimated. The metabolites recognized were the alcohol (II), aldehyde (III) and acid (IV) together with small amounts of the diphenyl ethane (VI). Three other metabolites appear to be present in relatively large amounts. The pattern of metabolites in bile and urine has been compared. It is suggested that the relatively low excretion of the acid (IV) in the urine compared to bile is due to a selective reabsorption of this compound after biliary excretion.

IN a previous paper, Ladomery, Ryan & Wright (1967) described the excretion of small doses (100 μg) of butylated hydroxytoluene (BHT; I) in the rat. They found that BHT was excreted slowly in urine and faeces because of a rapid enterohepatic circulation. This indicated that BHT was being rapidly metabolized since BHT itself is a non-polar molecule which would not be expected to be excreted by the liver unchanged. This paper describes the detection and estimation of the biliary metabolites from rats given small doses of [$^{14}$C]BHT. Previous metabolic work on BHT (Dacre, 1961; Akagi & Aoki, 1962) is summarized in Fig. 1.




From the Department of Pharmacy, University of Sydney, N.S.W., Australia.

## Experimental

### MATERIALS

[$^{14}$C]3,5-*Di-t-butyl-4-hydroxytoluene* (2,6-di-t-butyl-*p*-cresol; [$^{14}$C]BHT) was purchased from New England Nuclear Corp., Boston, Mass., U.S.A. It was used as 0·02% solution in 50% aqueous ethanol.

3,5-*Di-t-butyl-4-hydroxybenzoic acid* (IV) was prepared by passing isobutylene into a well-stirred mixture of methyl *p*-hydroxybenzoate (6·16 g) and concentrated sulphuric acid (12·0 g) at 100°. When the theoretical quantity of gas (4·5 g) had been taken up (about 36 hr), the mixture was extracted with chloroform-light petroleum (b.p. 60–80°) (1:1, 150 ml) and the solution washed with 10% aqueous sodium hydroxide (4 × 50 ml). The organic phase was evaporated to give methyl 3,5-di-t-butyl-4-hydroxybenzoate (2·0 g), m.p. 167–168° from methanol. Dacre (1961) reports m.p. 165°.

The ester was hydrolysed by refluxing with an excess of 0·5N methanolic sodium hydroxide under $N_2$ for 4 hr. Dilution with water and acidification precipitated the acid, m.p. 219–220° from light petroleum (b.p. 60–80°). Dacre (1961) reports 210°; Yoke, Dunbar & others (1956) report m.p. 218°.

3,5-*Di-t-butyl-4-hydroxybenzaldehyde* (III) was prepared by the method of Campbell & Coppinger (1953), m.p. 189° (lit., m.p. 189°). The semicarbazone had m.p. 227° from ethanol. Found: C, 65·8; H, 8·3; N, 14·2%. $C_{16}H_{25}N_3O_2$ requires C, 66·0; H, 8·6; N, 14·4%.

3,5-*Di-t-butyl-4-hydroxybenzyl alcohol* (II) was prepared by reduction of the aldehyde with sodium borohydride, m.p. 145–146° from methanol or light petroleum (b.p. 60–80°) (Bolle & Tomaszewski, 1963, report 140°). Treatment of the alcohol with pyridine and acetic anhydride gave 3,5-di-t-butyl-4-hydroxybenzyl acetate, m.p. 99–100° from light petroleum (b.p. 60–80°). Adingi & Acid (1962) report m.p. 98–99°.

1,2-Di(3,5-di-t-butyl-4-hydroxyphenyl)ethane (VI) was prepared by hydrogenating 3,5,3′,5′-tetra-t-butylstilbene-4,4′-quinone (Cook, Nash & Flanagan, 1955) in ethanol at 25° and 200 lb/in$^2$ with Raney nickel. Evaporation of the ethanol gave the diphenylethane, m.p. 173–174° from aqueous acetone. (Cook, 1953, reports m.p. 174–175°).

### CHROMATOGRAPHY

Bile samples were spotted on Whatman No. 1 paper and chromatographed in n-butanol–water (1:1) (solvent A). Spots were visualized with Gibbs reagent. Radioactive areas were detected by scanning the paper chromatogram with a Vanguard model 880 Autoscanner. Substances eluted from the spots were hydrolysed with acid and the ether, soluble fraction chromatographed on Whatman No. 1 paper using cyclohexane–propanol (1:1) (solvent B). Spots were detected as before. These extracts were also chromatographed on thin-layers of silica gel with ether–light petroleum (1:1) (solvent C). Radioactive areas on thin-layer plates were detected by exposure to X-ray film.

METABOLIC EXPERIMENTS

Urine and bile were collected as described by Ladomery & others (1967). Hydrolyses were made by refluxing bile with 2N hydrochloric acid under nitrogen for 3 hr. Metabolites were extracted continuously using peroxide-free ether.

REVERSE ISOTOPE DILUTIONS

These were made by adding the required compound to bile. Determinations on unhydrolysed bile were made by adding ether (10 ml) and shaking well. The mixture was left standing at 3° for 48 hr with frequent shaking. The compound was re-isolated from the ether and purified to constant count. Hydrolysis experiments were made by refluxing the bile and reference compound with an equal volume of 4N hydrochloric acid. The hydrolysate was extracted with ether, the compound isolated and purified to constant count.

RADIOACTIVITY

This was assayed by liquid scintillation counting. Biological fluids and chromatogram eluates were accurately pipetted into scintillation fluid. Pure compounds were accurately weighed into vials and dissolved in the scintillator. Radioactive areas from thin-layer plates were removed and suspended in the scintillator. They were kept in the dark for 24 hr before counting.

## Results

Paper chromatography of rat bile containing the metabolites from an intravenous injection of [$^{14}$C]BHT is summarized in Tables 1 and 2. Six

TABLE 1. PAPER CHROMATOGRAPHY OF UNHYDROLYSED RAT BILE AFTER INTRAVENOUS INJECTION OF [$^{14}$C]BHT

| Unhydrolysed bile; solvent system A | | | |
|---|---|---|---|
| Spot | Rf value | % Radioactivity excreted | Compound Rf value reported* |
| A | 0·01 | 3·9 | |
| B | 0·03 | 4·3 | |
| C | 0·54 | 47·5 | BHT-hippuric acid 0·5–0·55 |
| D | 0·60 | | |
| E | 0·83 | 28·0 | BHT-ester glucuronide 0·3 |
| F | 0·95 | 15·1 | conjugated phenols 0·[illegible]–1·0 |
| Total | | 99·8 | 9[illegible]% of dose excreted |

* Dacre (1961).

radioactive areas were detected. Spot C, which overlapped with D, corresponded to the glycine conjugate* of 3,5-di-t-butyl-4-hydroxybenzoic

* Wright, Abintonwa & others have been unable to detect this compound as a metabolite of (II) and have questioned Dacre's (1961) findings. We do not wish to imply a positive identification of any biliary conjugate in this present paper.

TABLE 2. CHROMATOGRAPHY OF ELUTED AND ACID HYDROLYSED BILIARY METABOLITES IN SYSTEM B

| Spot hydrolysed | Rf value of hydrolysate | Reference compounds (Fig. 1) | Rf value |
|---|---|---|---|
| A | —* | IV | 0·49 |
| B | 0·06 | III | 0·67 |
| C + D | 0·13, 0·52, 0·81 | I | 0·85 |
| E | 0·47 | VI | 0·88 |
| F | 0·43, 0·70, 0·89 | | |

* Not detected after hydrolysis.

acid (IV) (47·5% of total activity) and spot E to its ester glucuronide (28%) (Dacre, 1961). Spot F was probably unconjugated phenols which amounted to about 16% of the total activity. This was confirmed by reverse isotope dilution (Table 3) which showed that the acid (IV),

TABLE 3. ESTIMATION OF [$^{14}$C]BHT METABOLITES IN RAT BILE AFTER INTRAVENOUS INJECTION OF [$^{14}$C]BHT

| Compound | % Dose in unhydrolysed bile | % Dose in hydrolysed bile |
|---|---|---|
| I | 0·5 | 0·5 |
| VI | 0·4 | 0·4 |
| II | 1·0 | 1·6 |
| III | 5·5 | 5·5 |
| IV | 4·4 | 39·5 |
| Total | 11·8 | 47·5 |

* 55% of administered radioactivity was excreted.

aldehyde (III) and alcohol (II), BHT dimer (VI) and BHT (I) together accounted for 12% of the biliary radioactivity before hydrolysis. Spots A, B and D were unidentified.

Elution of the radioactive areas from the paper chromatograms, acid hydrolysis and chromatography of the hydrolysates gave the results summarized in Table 2. Spot A was lost, spot B appeared to be unaltered. Spots C and D gave the acid (IV) and two unidentified spots. Spot E gave only the acid, while spot F gave the expected acid (IV), aldehyde (III) and dimer (VI). The alcohol (II) was probably in too low a concentration to be detected. Reverse isotope dilution studies (Table 3) of the bile hydrolysate showed that the acid (IV) was a major constituent, being nearly 40% of the total. The aldehyde (III), alcohol (II), BHT (I) and the dimer (VI) were minor constituents. Exactly half (47·5%) of the total activity was accounted for as these substances.

Because of a lack of reference conjugates it was decided to work with the bile hydrolysates. At this time it was found that better resolution of the biliary metabolites was achieved by thin-layer rather than paper chromatography. The chromatography of hydrolysed bile extracts after an intravenous injection of [$^{14}$C]BHT is shown in Table 4. Six radioactive areas were detected with X-ray film. Spot B did not appear in this bile, but was detected in "late" (72 hr) bile (see below). The relation between the unidentified compounds detected on thin-layer plates and on the paper

TABLE 4. THIN-LAYER CHROMATOGRAPHY OF ACID HYDROLYSED BILE AFTER AN INTRAVENOUS INJECTION OF [$^{14}$C]BHT (SOLVENT C)

| Radioactive spot | Rf value | % Dose excreted | Reference compound (see Fig. 1) | Rf value |
|---|---|---|---|---|
| A | 0·0 | 35·0 | | |
| B* | 0·1 | — | | |
| C | 0·25 | 37·1 | IV | 0·23 |
| D | 0·52 | 1·5 | II | 0·52 |
| E | 0·65 | 11·0 | | |
| F | 0·71 | 4·5 | III | 0·71 |
| G | 0·88 | 1·1 | VI } I } | 0·92 } 1·0 |
| Radioactivity residual | | 4·0 | | |
| Total† | | 94·2 | | |

* Metabolite B was found only in bile collected 72 hr after dosing.
† 95% of dose excreted in bile.

chromatograms is unknown. Spot C was the acid (IV), D the alcohol (II), F the aldehyde (III) and G was either BHT (I) or the dimer (VI). Spots A and E were unidentified.

This thin-layer technique was then applied to separate and estimate the metabolites appearing in bile collected for 6 hr immediately after an intraperitoneal injection ("early" bile) and also in the bile collected 72 hr after injection ("late" bile). The results are summarized in Table 5.

TABLE 5. ELUTION AND ESTIMATION OF [$^{14}$C]BHT METABOLITES ON THIN-LAYER CHROMATOGRAMS OF HYDROLYSED BILE AFTER INTRAPERITONEAL ADMINISTRATION

| Compound | % Radioactivity excreted | % Dose excreted |
|---|---|---|
| 0–6 hr bile (51·9% of dose excreted) | | |
| A | 33·8 | 17·5 |
| B | — | — |
| IV | 39·2 | 18·3 |
| II | 2·8 | 1·4 |
| E | 1·8 | 0·9 |
| III | 3·2 | 1·7 |
| VI } I } | 4·3 | 2·2 |
| Residual activity* | 13·4 | 7·0 |
| Total | 95·5 | 49·5 |
| 72–78 bile (11·6% of dose excreted) | | |
| A | 15·0 | 1·7 |
| B | 15·6 | 1·8 |
| IV | 52·6 | 6·1 |
| VI | 4·0 | 0·5 |
| Residual activity* | 11·1 | 1·3 |
| Total | 98·3 | 11·4 |

* Radioactivity remaining on plate after removal of radioactive areas.

After being revealed by exposure to X-ray film, the radioactive areas were scraped off the plates and counted.

In "early" bile the acid (IV) was the major component identified. The aldehyde (III), alcohol (II), BHT (I) and dimer (VI) accounted for only about 10% of the excreted radioactivity. Spot E was also a minor

component. Spot A, however, was present to the same extent as the acid (IV).

In "late" bile a simpler metabolic pattern appeared. Spot A was accompanied by an equal amount of a new spot, B. The acid (IV), was found and also a spot which, in view of the time elapsed since dosing, must have been the dimer (VI).

A complicating factor in this work was that not all the radioactivity spotted on to the plates was accounted for by the areas detected on X-ray film. A count of the activity of the residual silica gel showed this to contain the remaining radioactivity. The reason for this is not known. However, other workers in these laboratories (R. E. Thomas, private communication) have observed similar effects with tritiated cardiac glycosides and genins. The errors introduced by this retention of activity by the gel may be relatively small since there was good agreement between the reverse isotope dilution studies and elution from the plate. It is possible that part of the streaking of radioactivity is due to traces of decomposition products.

Vigorous acid hydrolysis caused loss of metabolite B from "late" bile (Table 6), but did not appreciably alter the amounts of the remaining metabolites. Table 6 also contains a comparison of pooled 4-day urine metabolites and "late" bile metabolites. It is interesting to note that the acid (IV) is not a major urinary constituent as it is in bile.

TABLE 6. ESTIMATION OF [$^{14}$C]BHT METABOLITES IN POOLED URINE AND LATE BILE HYDROLYSED WITH 6N HYDROCHLORIC ACID

| Compound (see Fig. 1) | 1–4 Day urine* | | 72–78 hr bile† | |
|---|---|---|---|---|
| | % Excreted radioactivity | % Dosed radioactivity | % Excreted radioactivity | % Dosed radioactivity |
| A | 29·2 | 6·3 | 15·9 | 1·7 |
| B | — | — | — | — |
| IV | 16·2 | 5·1 | 52·5 | 6·1 |
| II | 24·6 | 7·7 | — | — |
| E | — | — | — | — |
| III | 10·4 | 3·3 | — | — |
| I, VI | — | — | 6·0 | 0·5 |
| Residual activity | 24·0 | 7·2 | 20·1 | 2·3 |
| Total | 95·4 | 29·6 | 94·4 | 10·6 |

* 31–[illegible]% of dose excreted in urine.
† 11–[illegible]% of dose excreted in 72–78 hr bile.

## Discussion

The differing proportions of metabolites in "early" and "late" bile as well as in pooled 4-day urine suggest a selective process of biliary and urinary excretion. However, as yet, we have little information on the nature of the conjugates excreted in bile, and none on the nature of the compounds re-absorbed from the gut. In addition, nothing is known of the faecal metabolites. Yet it is interesting to speculate about the high proportion of the acid (IV) which appears in the bile. "Early" bile contains 34% and "late" bile 55% of the total excreted activity as (IV),

while pooled 4-day urine contains only 16%. The acid (IV) is mainly excreted as the ester glucuronide by rats (Wright & others, 1965). It would be hydrolysed by plasma esterases and therefore preferentially excreted by the liver. Ether glucuronides would probably be more stable to esterase action and would therefore be excreted more efficiently by the kidney. This assumption agrees with the minor amounts of the alcohol (II) found in bile and the high proportion (25%) in urine. More aldehyde (III) is found in "early" bile than pooled urine. It is possible that there is an equilibrium between the alcohol and aldehyde effected by the reversible alcohol dehydrogenase systems of liver during enterohepatic circulation.

It is interesting that Wright & others (1965) found a slow excretion of label from doses of 3,5-di-t-butyl-4-hydroxybenzyl alcohol[$^{14}$C] (II). Rats and dogs both excreted this compound in urine and faeces over several days. The major metabolite was the acid (IV) together with minor amounts of unidentified metabolites. In the light of the present work and its distribution studies, it is clear that the metabolites from (II) also undergo extensive enterohepatic circulation. Wright & others did not detect any unchanged (II) in the excreta.

Because of the agreement between the urinary excretion of tritiated BHT (Golder, Ryan & Wright, 1962) and [$^{14}$C]BHT (Ladomery, Ryan & Wright, 1963) the latter workers suggested that there might be little attack at the methyl group of BHT. It was known that 50% of the label in tritiated BHT was in the methyl group. It is now clear from the present work that, although the proportion of the acid (IV) is low in the urine, this is due to a process of selective excretion rather than selective oxidation.

Among the metabolites detected by Dacre (1961) was the alcohol, $\beta$-(3-t-butyl-2-hydroxy-5-methylphenyl)-$\beta\beta$-dimethylethanol, formed by oxidation of the t-butyl group. This compound does not seem to have been detected in the present work. It could not be isolated from rabbit urine using Dacre's (1961) method.

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# Effects of Butylated Hydroxytoluene on the Ultrastructure of Rat Hepatocytes*

*Bernard P. Lane, M.D., and Charles S. Lieber, M.D.*

Mammalian liver is a mitotically inactive tissue which has cells with a capacity for rapid alterations in response to increased demands upon its many metabolic functions. Although many changes in liver function have not been correlated with specific ultrastructural modifications, detoxification of some pharmacologic agents, including phenobarbital, nikethamide, tolbutamide, alcohol, and a variety of carcinogens, has been demonstrated to be associated with proliferation of agranular endoplasmic reticulum.[1, 6-12, 15, 17, 18, 20, 23, 24] This report describes the changes observed in rat liver after administration of butylated hydroxytoluene (BHT), an antioxidant widely used as food additive. BHT has been previously reported to result in increased liver weight,[5] but associated ultrastructural or biochemical changes were not known. It was found in the present study that the effects of this agent differ from those of other drugs in that repeated administration of BHT resulted not only in increases in hepatocytic agranular endoplasmic reticulum, but also in enhanced mitotic activity.

## MATERIALS AND METHODS

Thirty-six Sprague-Dawley rats, weighing 150 to 200 gm., were pair-fed nutritionally adequate liquid diets[10] with or without butylated hydroxytoluene over periods ranging from 1 to 24 days. The dose of BHT was 1.7 mg. per calorie the first day, 2.2 mg. per calorie the second day, and 2.8 mg. per calorie thereafter. An additional 18 rats received BHT, 1.4 gm. per kg. of body weight, in a single dose administered through a gastric tube and were then fed a control liquid diet.

Tissue was obtained for examination by electron microscopy at 1, 2, 3, 6, 7, and 24 days after the onset of ingestion of the agent in food. Liver specimens from each of three pairs of animals sacrificed at these times were studied. Groups of three animals from those receiving a single dose by gastric tube were sacrificed at 16, 24, and 48 hours, and specimens of the livers of these rats were also examined by electron microscopy. A control specimen was obtained from the litter mate of these animals, which received an identical diet except for the added BHT. Drug-treated and corresponding control rats were always sacrificed at the same time to eliminate the effect of diurnal variation. In all cases, small cubes of tissue were excised, fixed by immersion in 4 per cent gluteraldehyde in phosphate buffer at pH 7.4 for 24 hours, and then treated with unbuffered 1 per cent osmium tetroxide. The specimens were dehydrated with alcohol, embedded in Epon 812, and sectioned with an LKB Ultrotome. Sections stained with lead salts were examined with an RCA EMU-2E electron microscope. A section of tissue was also fixed in 10 per cent neutral formalin for processing for light microscopic examination. In each liver section 5000 cells were surveyed to assess the number of cells in mitosis. For statistical analyses, each individual result was compared to the corresponding control, and the mean of the

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From the Departments of Pathology and Anatomy, New York University School of Medicine, New York, New York; and the Liver Disease and Nutrition Unit, Cornell Medical Division, Bellevue Hospital, and the Department of Medicine, Cornell University Medical College, New York, New York. Dr. Lieber is a recipient of the United States Public Health Service Research Career Development Award K3-AM-22590.

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individual differences was tested by the Student *t*-test.

## RESULTS

All specimens from animals receiving BHT either in their diet or by a gastric tube exhibited a proliferation of smooth-surfaced endoplasmic reticulum within some hepatocytes. Aggregates of branching tubular systems occupied large areas of cytoplasm, to the exclusion of other organelles (Figs. 1 to 4). Whorl or "fingerprint" arrays were not noted and the diameter of tubules ranged from 500 to 800 Å. Little glycogen was noted within these aggregates of tubules, although glycogen-rich cytoplasmic areas were present to a normal extent in the animals on long term drug ingestion in the diet. Similarly, the other organelles were comparable, in number and appearance, to those in control specimens.

The proliferation of smooth endoplasmic reticulum was noted in 20 to 50 per cent of liver cells in the animals sacrificed after 1 and 2 days of ingestion of the drug, in 70 to 80 per cent of liver cells in animals receiving the drug for 3 to 7 days, and in more than 50 per cent of the hepatocytes in specimens from rats receiving the drug for 24 days. From 20 to 50 per cent of the cells in specimens from animals sacrificed 1 or 2 days after a single dose showed the change, and at 16 hours, scattered cells, ranging up to 15 per cent of those examined, exhibited proliferation of agranular endoplasmic reticulum. A zonal distribution within the hepatic lobule was not apparent in any of these groups.

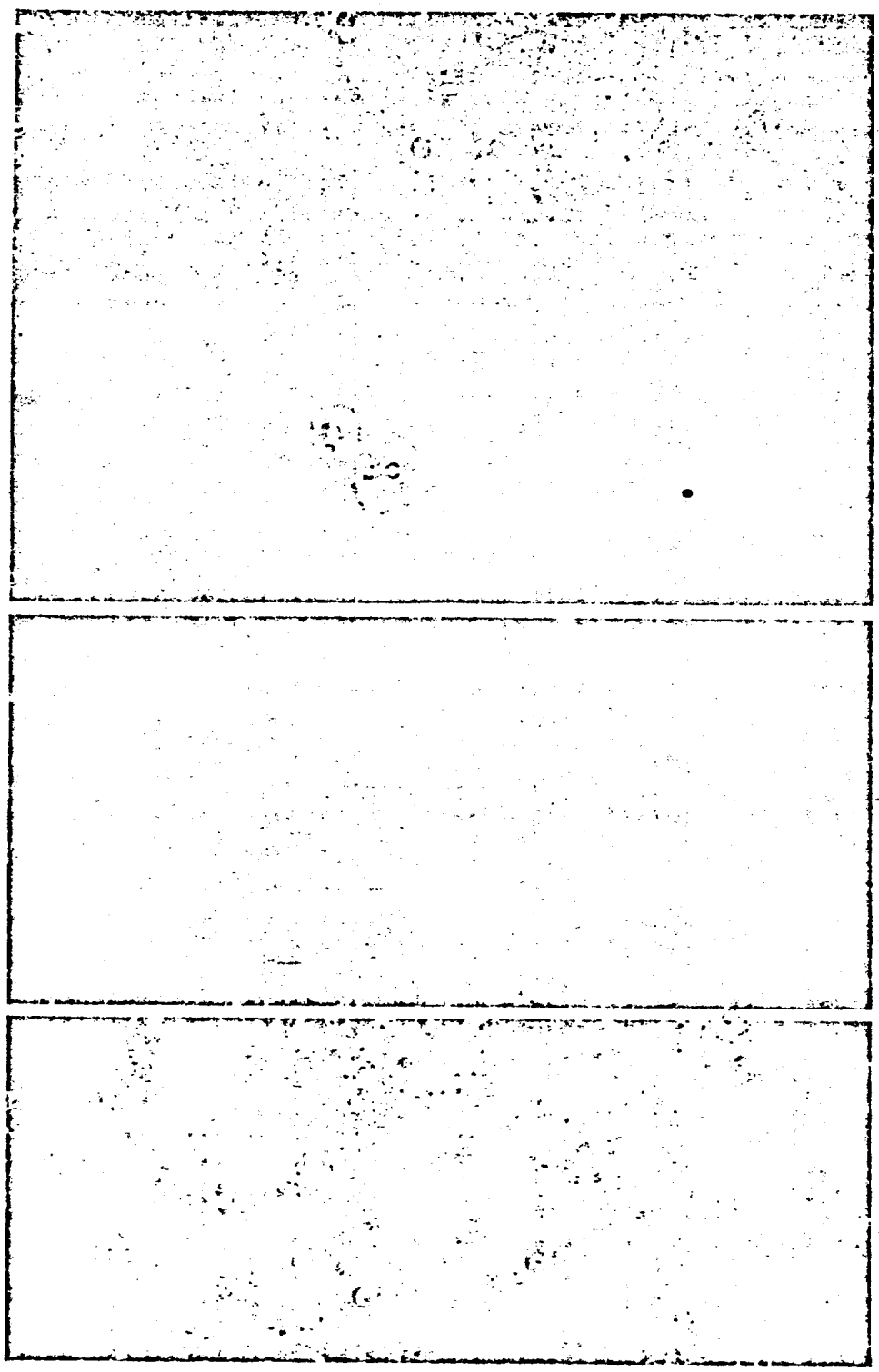
All specimens of liver obtained during the first 3 days of continuous drug ingestion or in the 2 days after administration of a single large dose via the gastric tube exhibited an increase in heterochromatin aggregated around the nucleus, when compared to control samples from untreated litter mates. The heterochromatin often formed a complete perinucleolar ring (Figs. 4 and 5). Heterochromatin aggregation, although less prominent, was also noted in samples obtained during the later periods studied. In all instances, the cells exhibiting perinucleolar chromatin aggregates also contained proliferated smooth endoplasmic reticulum, usually of a moderate degree. No definable alteration in nucleolar structure was discerned.

Increased mitotic activity was also noted in two-thirds of the samples of liver obtained after 1 to 4 days of continuous ingestion of BHT (Fig. 6). Although mitotic rates varied from negligible activity to 0.6 per cent, with an average of 0.2 per cent in each pair, values observed in the liver of the BHT-treated animal consistently exceeded the rate of the litter mate control specimen ($p < 0.05$). No period of peak activity was observed. Cells with nuclei in various stages of division always exhibited aggregates of proliferated agranular endoplasmic reticulum (Fig. 6). No increase in mitotic activity was observed in the rats given a single dose of BHT by gastric tube.

## DISCUSSION

A variety of parenterally and orally administered pharmacologic agents has been demonstrated to induce proliferation of agranular endoplasmic reticulum in hepatocytes. The structure of this increased agranular endoplasmic reticulum has varied from agent to agent, sometimes consisting largely of whorls of membranous channels or vesicular forms[1, 6, 7, 10, 12, 15, 17, 23, 24] and in other instances exhibiting a plexiform array of anastomosing tubules.[9, 15, 20] Many of the agents producing these ultrastructural alterations are carcinogens or cytotoxins and produce changes in other cytoplasmic organelles as well.[1, 6, 7, 11]

Phenobarbital and dimethylnitrosamine are two of the agents known to induce proliferation of smooth endoplasmic reticulum.[6, 9] Studies of the isolated hepatocytic agranular endoplasmic reticulum obtained from drug-treated animals have revealed a large increase in enzymes participating in degradation of these agents,[3, 21, 22] indicating that the administration of the drug induces the formation of membrane systems rich in the appropriate detoxifying enzymes. Although isolation of the microsomal fraction and assays for enzyme activity have not yet been performed on liver from BHT-treated animals, the form and distribution of agranular endoplasmic reticulum resemble that seen after phenobarbital treatment.



FIGS. 1 TO 3

Furthermore, as in the case of phenobarbital,[9] the livers of animals treated with BHT have exhibited an average of 50 per cent increases in wet and dry weight, as well as total nitrogen, in a matter of 3 to 4 days.[14] The mitotic rates observed after BHT ingestion were too small to account for this weight gain by increased cell numbers, and assays for glycogen and lipid content revealed no disproportionate increases.[14] These considerations suggest that the greater part of the increase is due to increased individual cell mass consisting in large measure of agranular endoplasmic reticulum.

The perinucleolar chromatin aggregation after BHT administration has not been described in studies with other agents, although an increase in nucleolar size has been noted in ultrastructure studies of the effects of thioacetamide and $\beta$-3-thienylalanine.[1, 11] This intranuclear alteration during the early period after administration of BHT suggests a role in the inductive process, but the mechanism has not yet been explored.

A marked increase in radioactive thymidine incorporation after BHT administration has been observed[14] with a parallel increase in the number of mitotic cells. The absence of a clear-cut peak of mitotic activity, however, suggests prolonged asynchronous division. This mitotic response is therefore probably different from the synchronous divisions noted in rat livers after partial hepatectomy. The ultrastructural changes, increased lysosomal activity, and cell surface alterations in individual hepatocytes after amputation of 70 per cent of the rat liver[2, 4, 13, 16] were not observed after BHT ingestion, which also indicates that the mechanisms are different. Other agents which cause proliferation of smooth endoplasmic reticulum without widespread cytotoxic effects have not been reported to result in increased mitotic activity. However, this mitotic activity, occurring asynchronously, may be due to a continuing stimulation to produce detoxifying apparatus beyond the capacity of the existing numbers of cells. The apparent virtual absence of mitotic activity in animals treated with a single large dose might be further evidence in support of this hypothesis.

The dose of BHT administered to rats in the present study greatly exceeds the amount ingested with an average American diet, and can be calculated to represent approximately 200 times the maximal allowable dose specified by the World Health Organization. Further investigations are therefore necessary before the present findings can be transposed to a population consuming BHT-containing diets.

The observation that large doses of BHT induce a change in nuclear and cytoplasmic morphology suggests a role for this agent in studies to correlate morphologic and biochemical events accompanying drug detoxification. The apparently asynchronous mitotic activity following BHT administration may also allow it to serve as a system for studies of the stimulation of cell replication in a normal, nearly amitotic tissue, thus circumventing some of the problems of disordered function encountered in studies of synchronous division following partial hepatectomy.

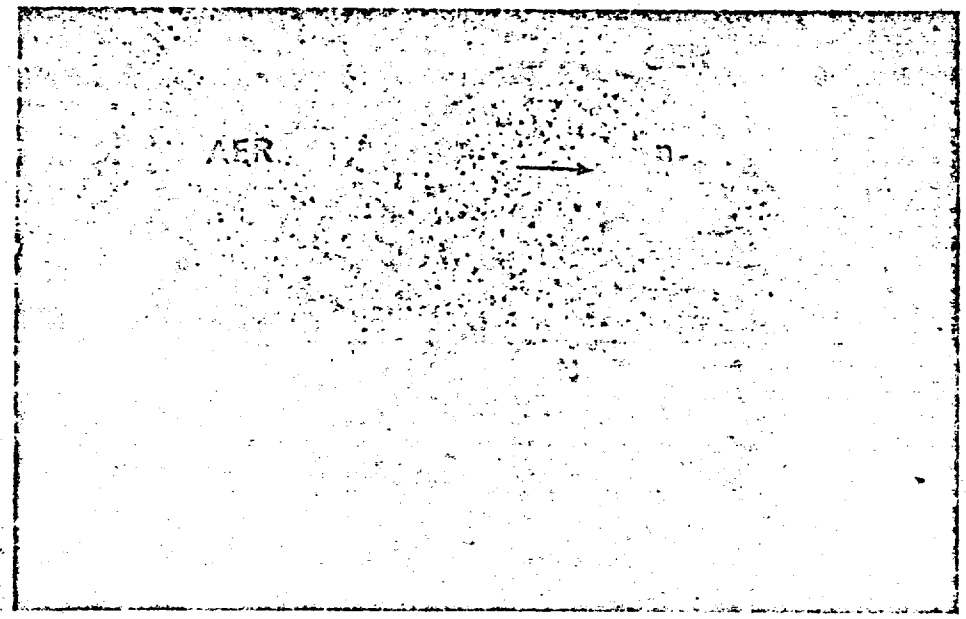
## SUMMARY

Butylated hydroxytoluene, an antioxidant widely used as food additive, when administered in large doses to rats resulted in significant hepatomegaly which was associated

FIG. 1. Electron micrograph of portions of two hepatocytes from a specimen obtained after 4 days of continuous ingestion of butylated hydroxytoluene (BHT) with the diet. Aggregates of agranular endoplasmic reticulum (*AER*) occupy much of the cytoplasm. *Bc*, Bile canaliculus; *N*, nucleus; *G*, Golgi apparatus. ×5,000.

FIG. 2. Higher magnification of proliferated agranular endoplasmic reticulum in rat liver after BHT ingestion for 3 days. The aggregates are composed of anastomosing tubular channels. Ribonucleoprotein particles (*arrows*) are scattered between membranous channels. ×20,000.

FIG. 3. Aggregate of agranular endoplasmic reticulum in a hepatocyte in a control specimen. The network of membranous channels is less tightly woven than that seen in specimens from BHT-treated animals. Glycogen rosettes (*Gly*) rather than ribonucleoprotein particles are present in clumps between the tubules. ×20,000.



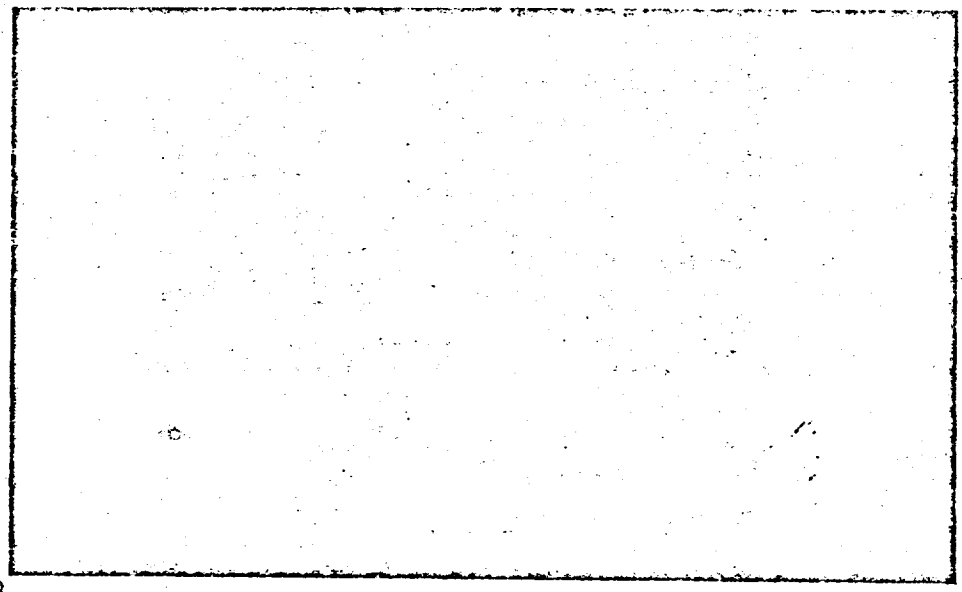



FIG. 4. Electron micrograph of a portion of a hepatocyte in a control specimen. The agranular endoplasmic reticulum (*AER*) consists of a loosely woven net with glycogen rosettes in its interstices. Unlike the cells in Figure 1, no large compact masses of agranular endoplasmic reticulum are present, and most of the cytoplasm is occupied by granular endoplasmic reticulum (*GER*). The nucleus (*N*) contains a nucleolus (*n*) which is apposed to a small heterochromatin aggregate on one side (*arrow*). *Bc*, Bile canaliculus; *G*, Golgi apparatus. ×5000.

FIG. 5. Electron micrograph of binucleate rat hepatocyte in a specimen obtained after 2 days of butylated hydroxytoluene ingestion reveals several nucleoli (*n*) surrounded by a rim of aggregated chromatin. Proliferating agranular endoplasmic reticulum was observed in other areas of this cell. ×5000.

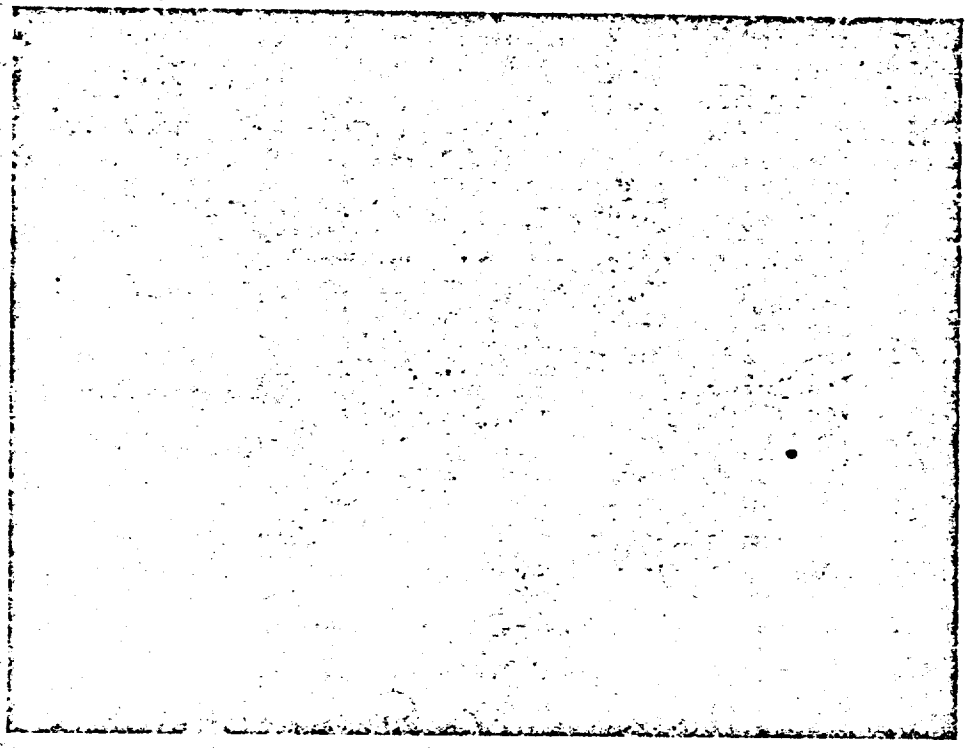


FIG. 6. Electron micrograph of an early metaphase cell in rat liver after 3 days of ingestion of butylated hydroxytoluene in diet. Aggregates of agranular endoplasmic reticulum (*AER*) surround the chromosomes (*C*). ×4000.

with marked proliferation of agranular endoplasmic reticulum, perinucleolar heterochromatin aggregation, and enhanced mitotic activity.

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## Reticulocytosis in Response to Dietary Antioxidants

**Abstract.** *Alpha-tocopheryl acetate, 1,2,dihydro-6-ethoxy-2,2,4-trimethyl quinoline, and butylated hydroxytoluene increased the number of circulating reticulocytes when added to the diet of chickens. Hematocrit values were not reduced and erythrocyte life-spans were not shortened by the antioxidants. The reticulocytosis is attributed to delayed loss of reticular material from the maturing erythrocytes.*

Reticulocytosis has been reported to occur in response to vitamin E and coenzyme $Q_4$ chromanol treatment of vitamin E–deficient monkeys, human infants, and children (*1*). The experiments described here demonstrate the antioxidants, when fed to chicks kept under normal dietary conditions, retard the maturation of reticulocytes.

In two experiments chicks were fed balanced diets based on natural ingredients and supplemented with antioxidants as indicated in Table 1. Retic-

ulocytes were counted by the slide method as previously described (2) after the chicks had been on experiment for several weeks. Hematocrit values were also determined. In a third experiment the effect of dietary antioxidants on the life-span of erythrocytes was studied by labeling in vivo with selenomethionine $^{75}$Se (3). This method estimates both production rate and survival time of erythrocytes. A different group of chicks was bled for each set of determinations of radioactivity in order to avoid the effect of blood loss on erythropoiesis which would result from repeated bleedings of the same birds. The hematocrit value of each sample was measured in order to relate radioactivity to packed cell volume. Reticulocytes were counted in the blood samples drawn 27 days after injection.

The reticulocyte counts of the chicks fed the control diets were similar to those previously reported for growing chicks (2). Supplementation of the diets with 1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline (EQ), butylated hydroxytoluene (BHT), and *dl*-alpha-tocopheryl acetate (TOC) increased reticulocyte counts significantly ($P < .01$) in all experiments. It should be noted that the levels of TOC fed were in excess of the requirement of growing chicks.

Hematocrit values were similar for the blood samples from the chicks fed the basal diets and the diets supplemented with antioxidants. It might accordingly be assumed that the observed reticulocytosis was not due to an insufficiency of red blood cells. This assumption was borne out by the results of the third experiment (Table 2) which indicate no acceleration of the rate at which red blood cells were either discharged into or removed from the circulation of the birds receiving antioxidants. Radioactivity of the fluid hemolysates from red blood cells of birds fed TOC was higher 27 days after injection of the tracer than in the case of the control birds, or the birds fed EQ. Although the markedly greater standard deviations of the values for the group treated with TOC make it difficult to calculate a valid estimate of the significance of the difference, it is apparent that erythrocyte turnover was slower in some of the birds treated with TOC.

It is emphasized that the reticulocytosis reported here occurred when antioxidants were fed to nutritionally normal animals in contrast to that reported in connection with alleviation of a vitamin E deficiency (1). In the present experiments the control chickens were not deficient in vitamin E and the antioxidants were administered for several weeks prior to counting of reticulocytes. The reticulocytosis is attributed to retardation of the maturation process of the cells rather than to acceleration of erythropoiesis. Reticulocytes contain more phospholipid than do mature cells (4), and there is other evidence that cell aging is accompanied by a decrease in total lipids, cholesterol, and phospholipids (5). Dietary antioxidants have been shown to moderate the adverse effects of exposure to hyperoxia (6), and could therefore be expected to retard autoxidation of tissue lipids under conditions of normal oxygen concentration.

If the disappearance of reticular material is related to the decline in lipids in maturing red blood cells and involves autoxidation of the lipids, delay of the maturation process could result from antioxidant protection of the lipid components.

Table 1. Effect of dietary antioxidants on reticulocyte count and hematocrit value. Results are given as means ± standard deviation. In experiment 1, eight male White Leghorn chicks per treatment were placed on test at 8 weeks of age; in experiment 2, ten male White Leghorn chicks were on each treatment from 0 to 4 weeks of age. BHT, butylated hydroxytoluene; EQ, 1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline; and TOC, *dl*-alpha-tocopheryl acetate.

| Treatment | After 2 weeks | After 3 weeks | |
|---|---|---|---|
| | Reticulocytes (%) | Reticulocytes (%) | Hematocrit (%) |
| | *Experiment 1* | | |
| Control | 11.8 ± 2.0 | 13.8 ± 2.3 | 30.8 ± 2.1 |
| BHT (0.5 g/kg) | 11.9 ± 2.5 | 19.2 ± 5.5 | 30.9 ± 2.5 |
| BHT (5.0 g/kg) | 16.7 ± 3.8 | 19.3 ± 7.1 | 30.9 ± 2.3 |
| EQ (0.5 g/kg) | 18.5 ± 4.6 | 23.0 ± 5.3 | 30.1 ± 2.2 |
| EQ (5.0 g/kg) | 29.6 ± 6.9 | 46.7 ± 12.0 | 28.3 ± 3.3 |
| TOC (120 unit/kg) | 20.7 ± 3.3 | 31.6 ± 6.8 | 30.5 ± 3.1 |
| | *Experiment 2* | | |
| Control | 12.5 ± 2.1 | | 28.0 ± 1.9 |
| EQ (0.5 g/kg) | 16.3 ± 2.4 | | 27.8 ± 4.7 |
| EQ (5.0 g/kg) | 18.6 ± 4.6 | | 27.3 ± 5.7 |
| TOC (220 unit/kg) | 18.7 ± 2.0 | | 29.8 ± 2.1 |

Table 2. Erythrocyte turnover in chicks fed antioxidants. Male White Leghorn chicks were fed a balanced diet supplemented as indicated from 0 to 7 weeks of age. They were then injected intravenously with 1 $\mu$c of $^{75}$Se-labeled L-selenomethionine. Their red cells were tested 2, 9, and 27 days later for $^{75}$Se (count/min), and the percentage of reticulocytes was determined on day 27. Results are average counts per minute ± standard deviation of fluid lysates from 1 ml of packed cells. EQ, 1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline; TOC, *dl*-alpha-tocopheryl acetate.

| Treatment | Turnover after injection | | | |
|---|---|---|---|---|
| | Day 2* (count/min) | Day 9† (count/min) | Day 27† | |
| | | | (count/min) | Reticulocytes (%) |
| Control | 302 ± 63 | 926 ± 194 | 579 ± 61 | 11.8 ± 2.5 |
| EQ (2.5 g/kg) | 252 ± 119 | 949 ± 269 | 554 ± 96 | 16.9 ± 3.0 |
| TOC (220 unit/kg) | 308 ± 95 | 887 ± 316 | 784 ± 243 | 18.6 ± 5.1 |

* Twenty chicks per treatment. † Ten chicks per treatment.

B. E. MARCH
VIONA COATES
JACOB BIELY

*Department of Poultry Science, University of British Columbia, Vancouver, Canada*

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7. Supported by a grant from the National Research Council of Canada. We thank Monsanto (Canada) Ltd. for 1,2,dihydro-6-ethoxy-2,2,4-trimethylquinoline (Santoquin) and Hoffman-La Roche Ltd., Montreal, for *dl*-alpha-tocopheryl acetate used in the study.

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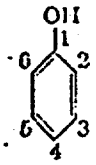
# Comparative Toxicity of Certain *t*-Butyl Substituted Cresols and Xylenols*

By W. A. McOMIE, HAMILTON H. ANDERSON, and F. M. ESTESS

Of the compounds studied, 2-*t*-butyl 4-methylphenol, 2-*t*-butyl 5-methylphenol, 2,4-di-*t*-butyl 5-methylphenol, 2,6-di-*t*-butyl 4-methylphenol and 3,5-dimethylphenol have a lower degree of acute toxicity than phenol itself. However, 2,4-dimethyl-6-*t*-butylphenol is unique in its toxicity to the rabbit, being as lethal by simple application to the intact skin as by the oral route of administration. This is apparently a species susceptibility since rats and guinea pigs are less susceptible to the compound by both the oral and the skin route. It is concluded that the dialkyl phenols are more irritant locally than the trialkyl phenols studied.

THE COMPOUNDS of chief interest[1] in this study are important as oxidation inhibitors. They are all derivatives of phenol and for sake of brevity will be designated by the position of the alkyl groups on the ring relative to the phenolic hydroxyl group according to the following scheme.




2-*t*-Butyl 4-methylphenol (2B4M)
2-*t*-Butyl 5-methylphenol (2B5M)
2,4-Di-*t*-butyl 5-methylphenol (24B5M)
→ 2,6-Di-*t*-butyl 4-methylphenol (26B4M)
6-*t*-Butyl 2,4-dimethylphenol (24M6B)
3,5-Dimethylphenol (35M)

Parallel experiments were conducted in certain instances with phenol, carvacrol, and thymol.

All of the compounds contained less than 1 per cent of impurities. Carvacrol, 2B5M and 24M6B were liquids at 25°. The others were solids. The physical properties of several compounds were reviewed by Pardee and Weinrich (1).

The purpose of this study was to determine the relative local and systemic toxicities of the compounds and to estimate their relative hazard to man.

## EXPERIMENTAL

**Intragastric Administration to Mice.**—Cottonseed oil solutions (1–4%) of the phenols were administered to previously fasted (sixteen to twenty-four hours) stock white, male, mice (weighing from 15 to 25 Gm. each) by means of a blunt-tipped 18-gauge needle attached to a syringe. Volumes given varied from 0.2–0.8 cc. (Table I).

After intragastric administration of cottonseed oil solutions of all compounds other than phenol; the mice reacted in essentially the same manner. There usually was depression to the point of prostration at the higher dose levels. At the lower dose levels no effect other than depression was observed. This was the general observation after administration of 2B4M, 2B5M, 24B5M, 24M6B, 26B4M, 35M, and thymol. Phenol in doses as low as 280 mg./Kg. caused all mice to exhibit muscular tremors and generalized body twitchings. Deichmann and Witherup (3) described these tremors or twitchings in rats, rabbits, and cats after phenol administration. These authors also found that *o*-, *m*-, and *p*-cresols cause these twitchings but were less severe with the cresols than with phenol itself. From our results it would appear that further substitution in the cresol molecule causes this effect to disappear.

The pathologic effects produced in mice by these compounds consisted of irritant action on the gastroenteric tract as evidenced by hemorrhages in the region of the small intestine. Severe edema and congestion of the lungs were usually found grossly after both acute and delayed deaths. There was no microscopic evidence of myocardial damage. Delayed deaths occurred after exposures to some of these compounds. An observation period of ten days was chosen arbitrarily. Some animals died as long as ten days after administration of the compounds. Phenol, 35M, and thymol were exceptions to this delayed-death effect. In no instance after application of these three compounds did death occur after forty-eight hours. The tri-substituted compounds, however, were particularly noteworthy in this delayed death effect. In the case of 24M6B and 26B4M over 50% of the deaths occurred ninety-six hours after application of the compounds. Livingston (4) found that delayed deaths (five days and more after administration) took place with carvacrol and thymol after intragastric administration to rabbits.

**Administration to the Skin of Rabbits.**—The liquid compounds (2B5M, 24M6B, and carvacrol) were placed in rubber cuffs and the solid compounds on gauze held in place by a flexible wire screen according to the method of Draize, *et al.* (5). In the case of the liquid compounds the exposures were for six hours. The severe local effects of 2B5M and carvacrol were evident after this time and 24M6B was lethal. All of the solids were allowed to remain on the skin for twenty-four hours (Table II). The compounds were also applied in ether by flowing the ether solution on to the shaved back of the rabbit (Table III).

From the above results the following generalizations were made concerning these compounds after administration topically to rabbits:

* Received September 20, 1948, from the Division of Pharmacology and Experimental Therapeutics, University of California Medical School, San Francisco 22, Calif.
[1] Supplied by the Shell Development Co., Emeryville, Calif., who aided in the support of this study.

TABLE I.—ACUTE INTRAGASTRIC TOXICITY OF ALKYL PHENOLS IN COTTONSEED OIL SOLUTION TO MICE

| Dose, Mg./Kg. | Mortality Ratio (10 Days' Observation) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 2B4M | 2B5M | 24B5M | 24M6B | [illegible] | 35M | Thymol | Phenol |
| 280 | 0/10 | ... | ... | 3/10 | ... | 0/10 | ... | 0/5 |
| 420 | 1/10 | ... | ... | 4/5 | 1/10 | 1/10 | ... | 0/5 |
| 620 | 7/10 | 2/5 | 1/10 | 5/10 | 4/10 | 4/10 | 0/5 | 4/5 |
| 940 | 5/10 | 1/10 | 2/10 | 4/5 | 4/10 | 9/10 | 0/5 | 5/5 |
| 1400 | ... | 6/10 | 3/10 | ... | 6/10 | ... | 3/10 | ... |
| 2100 | ... | 5/5 | 9/10 | ... | ... | ... | 3/5 | ... |
| $LD_{50}$[a] | 700 | 1080 | 1420 | 530 | 1040 | 620 | 1800 | 640[b] |
| Standard[a] Error $LD_{50}$ | ±35 | ±93 | ±108 | ±36 | ±204 | ±52 | ±224 | ... |

[a] Graphic method of Miller and Tainter (2) was used to obtain the approximate $LD_{50}$'s and their standard errors.
[b] Estimate.

TABLE II.—CUFF EXPOSURES OF RABBITS TO ALKYL PHENOLS

| Compound | Dose[a] | Duration of Exposure, Hr. | Effects Noted |
|---|---|---|---|
| Carvacrol | 2.7 | 6 | Immediate irritation, becoming erythematous. Leather-like appearance within 4 hr. Complete necrosis and eventual sloughing and scar formation. Died 72 hr. after exposure. |
| 2B4M | 2.2 | 24 | Similar local effects as with carvacrol. Died 72 hr. after exposure. |
| 2B5M | 2.9 | 6 | Similar local effects as with carvacrol. No apparent systemic effect other than that due to severe burn of the skin. Survived. |
| 2B5M | 3.9 | 6 | Similar picture as above. Survived. |
| 35M | 2.6 | 24 | Similar effect as with 2B5M. Survived. |
| 24B5M | 4.5 | 24 | Only slight irritation of skin (mild erythema without subsequent necrosis). No apparent systemic effect. |
| 26B4M | 3.3 | 24 | No evidence of skin irritation. No apparent systemic effects. |
| 24M6B | 2.9 | 6 | Died within 6 hr. from beginning of exposure. No local erythema or edema. Lung grossly edematous and congested. |
| 24M6B | 3.9 | 6 | Same effect as above. Both animals had sufficient lung edema to have caused death. |

[a] Dose is given in cc./Kg. for the liquid compounds (2B5M, carvacrol, and 24M6B) and in Gm./Kg. for the solid compounds. One rabbit was used at each dose level.

TABLE III.—APPLICATION OF ETHER SOLUTIONS OF ALKYL PHENOLS TO THE SKIN OF THE RABBIT

| Compound | Dose, Gm./Kg. | Effects Noted |
|---|---|---|
| Phenol[a] | 0.42 | Skin turned parchment-like within 24 hr. No apparent systemic effect. Complete necrosis of superficial layers after 10 days. Survived. |
| Thymol | 0.42 | Similar picture to that of phenol. |
| 35M | 0.42 | Similar picture to that of phenol. |
| 2B4M | 0.42 | Similar picture to that of phenol. |
| 2B5M[b] | 0.42 | Similar picture to that of phenol. |
| 24B5M | 0.42 | Some erythema and superficial sloughing but no severe tanning effect as seen with phenol. |
| 26B4M | 0.42 | Similar picture to that of 24B5M. |
| 24M6B | 0.08 | Died 35 hr. after application. No local effect. Edematous lungs found upon gross autopsy. |

[a] Liquefied phenol, U. S. P. XIII, was used.
[b] Given undiluted.

1. 2B4M, 2B5M, 35M, and carvacrol are severely irritant and resemble phenol in their coagulative effect on the skin. Carvacrol and 2B4M may cause fatalities through exposure of relatively large areas of the skin. This may be due to secondary effects from the severe chemical burn rather than from systemic absorption of the compounds themselves.

2. Although 24M6B is only mildly irritant it is readily absorbed from the skin and causes death through systemic action, rather than indirectly from skin injury.

3. The two solid tri-substituted alkylated phenols (24B5M and 26B4M) are the least irritant, and evidently little is absorbed from the solid form.

4. None of the other compounds had any apparent systemic effect after topical applications of a dose 5 times greater than the lethal dose of 24M6B (see Table V).

Effect of Alkyl Phenols on the Eyes of Rabbits.—Table IV summarizes the results of exposure to the eyes of rabbits. In each case 0.03 cc. of the solution was applied directly to the cornea.

The irritation to the rabbit's eye of these compounds places them in similar categories as shown previously for their action on the skin. Thymol, 35M, 2B4M, and 2B5M are all extremely irritant; 24B5M and 24M6B are less irritant but still dangerously irritant to mucous surfaces. 26B4M, due to

its lack of solubility [illegible] miscible solvent. Its lack of irritation in diallyl phthalate (6) might indicate that it had a much greater affinity for the relatively bland medium in which it was dissolved rather than for the corneal or conjunctival membrane. Allowing for this inability to demonstrate irritation in the case of 26B4M, it is apparent that all the compounds are injurious to the eye but are not particularly more so than thymol.

guinea pigs and rats by simple application without rubbing. The lethal doses obtained by this method are shown in Table V.

In rabbits there was a remarkable paralleling of toxicity by the oral and skin routes of administration. However, the rat and the guinea pig are not only less susceptible than the rabbit to intragastric administration of the compound but also show less likelihood of toxic effects through skin absorption

TABLE IV.—APPLICATION OF 0.03 CC. ALKYL PHENOLS TO THE RABBIT CORNEA

| Compound | Concentration | Result[a] in 1 Hr. | 24 Hr. | 72 Hr. | Fluorescein[b] Test, 24 Hr. |
|---|---|---|---|---|---|
| 2B4M | 40% in ethylene glycol | 31 | 80[c] | 80[c] | [d] |
| [illegible] | [illegible] | 32 | 78 | 80[c] | 100 |
| [illegible] | [illegible] in [illegible] glycol | 13 | 9 | 2 | 90 |
| [illegible] | [illegible] | 0 | 43 | 53 | 90 |
| 26B[illegible] | 40% in diallyl phthalate | 0 | 0 | 0 | 0 |
| 35M | 40% in ethylene glycol | 31 | 80[c] | 80[c] | 90 |
| Thymol (control) | 40% in ethylene glycol | 32 | 80[c] | 80[c] | 100 |

[a] Score of Draize, *et al.* (5), used (1944); the larger the number, the greater the injury to the mucous membranes.
[b] Numbers under fluorescein refer to percentage staining of corneal surface with fluorescein and indicate extent of damage to corneal epithelium.
[c] After 24 hr. the eye was closed so completely that injury could not be evaluated by the scoring method. The maximum score possible (110) was approached in the case of 2B4M, 2B5M, 35M, and Thymol. Permanent clouding of cornea resulted in case of 24M6B.
[d] Eye was completely closed and could not be opened to instill the fluorescein.

TABLE V.—COMPARATIVE LETHAL DOSES OF 24M6B TO RABBITS, RATS, AND GUINEA PIGS BY THE ORAL AND SKIN ROUTES OF ADMINISTRATION

| | Oral | | | Skin | | |
|---|---|---|---|---|---|---|
| | Rabbits | Rats | Guinea Pigs | Rabbits | Rats | Guinea Pigs |
| Lethal dose,[a] cc./Kg. | 0.055 | 1.4 | 0.42 | 0.055 | 10.7[b] | 7.1 |

[a] Lethal dose as defined by Deichmann and LeBlanc (7) as first lethal dose in the series.
[b] No deaths occurred. This was highest amount given.

**Comparative Toxicity of 24M6B by Skin and Oral Routes of Administration.**—The results of skin application to rabbits indicated that 24M6B had a systemic toxicity considerably above any of the other compounds when applied by this route. In addition it was not as irritant as some of the other compounds possibly rendering its hazard even greater since lack of irritation would not provide warning and could lead the person exposed to a false sense of security. Accordingly, a study of the comparative toxicity to rabbits, rats, and guinea pigs by the oral and skin routes of administration was made. The method of Deichmann and LeBlanc (7) was used for determining the approximate lethal doses by these routes. This method consists briefly of giving a single animal a dose differing by 50% from the preceding dose given to another animal. The approximate lethal dose by their definition is the lowest dose of the series that kills. These authors showed that for a group of compounds this approximate lethal dose varied from the $LD_{50}$ by about ±30%. Employing this method (with the exception that in some cases more animals were employed at each dose in the lethal range), the following results were obtained. Compounds were given orally either undiluted or dissolved in propylene glycol. Topical applications were made to the previously clipped area of the backs of guinea pigs and rabbits and to the abdominal surface of rats. The undiluted compound or other solutions of each (when small amounts were applied) were given to

The rat, in particular, is quite resistant by both the oral and the skin routes.

The signs of toxicity in rabbits were, first, depression, then dyspnea accompanied by râles and frothing at the nostrils. Convulsions ensued terminally probably due to asphyxia as the result of profound lung hemorrhage and edema, easily seen grossly at autopsy.

Signs of poisoning in rats and guinea pigs were essentially the same as in rabbits. The lung changes were not as severe as in rabbits even with lethal doses. The stomach wall and intestines were highly inflamed in both guinea pigs and rabbits after oral administration of the undiluted compound or as a 10% solution in propylene glycol. The evidence on species difference in susceptibility to 24M6B seems unmistakable. An indication that other compounds were not as toxic as 24M6B to the rabbit was seen when single rabbits survived intragastric doses of 0.62 Gm./Kg. of 26B4M and 0.94 Gm./Kg. of 2B5M, respectively, without any apparent effect. Both of these amounts are more than 10 times the intragastric $LD_{50}$ for rabbits of 24M6B.

## DISCUSSION

Aside from the previous communication on the toxicity of 24M6B by Treon and Cyama (8) no other data concerning the toxicity of these compounds have appeared in the literature. Related alkyl

phenols have been investigated by Lamson, *et al.* (9, 10) and studies have been made comparing the toxicity of thymol and carvacrol by Livingston (4) and Caujolle and Franck (11). Deichmann and Witherup (3) have compared the toxicity of phenol and the *o*, *m*-, and *p*-cresols.

The substituted cresols and xylenols studied, with the exception of 24M6B, in the rabbit, are more comparable in toxicity to thymol than to phenol itself. It would seem, in view of the widespread use of thymol in pharmaceutical products, that these compounds should present no particular hazard if used with caution.

The unique toxicity of 24M6B in the rabbit is of interest because its lethal dose by the skin and oral routes is nearly identical. The mechanism of this toxicity and the reason for its ease of penetration through the intact rabbit skin are as yet unexplained. This compound should be treated with utmost caution in view of its extreme toxic effects in the rabbit.

## SUMMARY

1. The range of acute intragastric $LD_{50}$ for mice of cottonseed oil solutions of certain *t*-butyl substituted compounds was from 530–1800 mg./Kg. The relative order of toxicity by this route from most toxic to least toxic was 6-*t*-butyl 2,4-dimethylphenol, phenol, 3,5-dimethylphenol, 2-*t*-butyl 4-methylphenol, 2,6-di-*t*-butyl 4-methylphenol, 2-*t*-butyl 5-methylphenol, 2,4-di-*t*-butyl 5-methylphenol, and thymol. The compounds may be considered as of intermediate toxicity between phenol and thymol in so far as the intragastric route for mice is concerned.

2. Carvacrol, thymol, phenol, 2-*t*-butyl 4-methylphenol, 2-*t*-butyl 5-methylphenol, and 3,5-dimethylphenol were severely irritant to the skin of rabbits upon prolonged exposure. The trisubstituted compounds (2,6-di-*t*-butyl 4-methylphenol, 2,4-di-*t*-butyl 5-methylphenol, and 6-*t*-butyl-2,4-dimethylphenol) while irritant are not in the same category as the dialkyl derivatives or phenol itself.

3. The compound 6-*t*-butyl 2,4-dimethylphenol, though only mildly irritant to the skin of rabbits, caused death in this species with as small a dose as 52 mg./Kg. applied to the skin. The $LD_{50}$ of this compound to rabbits is approximately the same by either the oral or the skin routes. Rats and guinea pigs, however, were less susceptible to its toxic action after intragastric administration and much less susceptible after percutaneous application.

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# Effects of the Food Additive Butylated Hydroxytoluene on Monolayer Cultures of Primate Cells

by
SUSAN M. MILNER
Department of Pathology,
University of Cambridge

Inhibition of monkey kidney cell numbers by the antioxidant food additive, butylated hydroxytoluene, is caused by a rapid depression of cellular metabolism. This effect varies according to sex and may be the result of more rapid metabolism of butylated hydroxytoluene in male cells.

AUTOXIDATION of unsaturated fatty acids involves the formation of free radicals during the induction period leading to the breakdown of carbon–carbon double bonds and consequent rancidity. Butylated hydroxytoluene (BHT), or 3,5-ditertbutyl-4-hydroxytoluene, acts as an antioxidant by accepting free radicals[1]. The onset of rancidity in foods containing lipids may therefore be delayed by the addition of BHT. The safety of BHT as a food additive remains suspect. It is not included on the list of permitted antioxidants in many countries[2], despite pathological, teratogenic[3,4] and biochemical tests[5–8] which indicate that BHT is a harmless compound in low doses.

Rats are the animals most frequently used for *in vivo* studies of BHT, and the oral dose required to cause an increase in relative liver weight and in the concentrations of drug metabolizing enzymes and urinary ascorbic acid is about 500 mg/kg body weight. It has, however, long been recognized that experimental animals other than rodents are required for toxicity studies of compounds for human consumption[9] because of species differences in the type and rate of metabolism[10,11].

In view of the questionable status of BHT as a food antioxidant, the *in vivo* results, and a possibility of species specific metabolic differences, it was considered important to examine the effects of BHT on primate cells. Primary cultures of monkey kidney (*MK*) cells were chosen rather than cell lines or strains in order to study cells as closely as possible to the *in vivo* state.

Initial experiments were designed to find the dose of BHT which stopped cell numbers increasing. When this had been established a more detailed investigation was carried out to study the effects of this dose on primate cell metabolism by measuring the rate of incorporation of radioactive precursors into DNA, RNA and protein.

Solutions of pure grade BHT, the metabolites 3,5-ditertbutyl-4-hydroxybenzoic acid (BHT-acid), 3,5-ditertbutyl-4-hydroxybenzyl alcohol (BHT-alcohol) and α-tocopherol were prepared fresh for each experiment by dissolving overnight in dimethylsulphoxide (DMSO). All dilutions were made up to contain 0·9 per cent DMSO with 199 tissue culture medium containing 10 per cent calf serum and antibiotics. For the radioactive precursor studies, solutions containing 0·1 μc./ml. of tritiated thymidine-6-T (*n*) ($^3$HTdR), 0·1 μc./ml. tritiated uridine-5-T ($^3$H-UR), and 0·05 μc./ml. D-L-leucine-1-$^{14}$C ($^{14}$C-leucine) were made up in phosphate buffered saline (PBS). Eight day old primary monolayer cultures of kidney cells obtained from 2 yr old rhesus monkeys were treated with 0·25 per cent trypsin at 37° C for 5 min and resuspended in growth medium to the order of $5 \times 10^4$–$10^5$ cells/ml. Constant stirring maintained a good suspension while aliquots (4 ml.) were removed and seeded into 2 in. diameter plastic Petri dishes. The cells were grown overnight at 37° C in an atmosphere of 5 per cent carbon dioxide in air.

At time zero the supernatant medium was removed, and the monolayers were covered by 4 ml. of growth medium (controls), 4 ml. of growth medium containing 0·9 per cent DMSO (DMSO controls), or 4 ml. of growth medium containing the required dilution of test compound in 0·9 per cent DMSO (test). All media were prewarmed to 37° C, and duplicate cultures were treated for each estimation. To avoid complications caused by individual animal variation, each complete experiment utilized cells from one animal only. After treatment for the required time intervals, the monolayers were labelled for 20 min at 37° C. They were then washed with ice-cold PBS, treated with versene, collected into test tubes and resus-

pended in a known amount of PBS. The cells in each suspension were counted, and 1 ml. was transferred to a small centrifuge tube for extraction. Perchloric acid (0·2 normal) was used to remove nucleotides from the samples, and the acid precipitated proteins and nucleic acids were dehydrated by two washes of ethanol before rendering them soluble in 0·3 ml. of hyamine at 60° C for 30 min[12]. The hyamine was dissolved in 3 ml. of scintillation fluid containing 0·01 per cent POPOP and 0·4 per cent PPO in toluene and was then pipetted into a counting vial. The samples were counted for 10 min in a Packard 'Tri-Carb' scintillation spectrometer. Precursor (0·8 ml.) was added to 3 ml. of Bray's scintillation fluid[13] and c.p.m. were measured for each batch of radioactive precursor prepared. Dividing the c.p.m. of $10^4$ cells by the c.p.m. of the precursor used and multiplying by 100 gave a value proportional to the percentage of precursor uptake into $10^4$ cells. Using this value a direct comparison between different experiments was possible.

The *MK* cell monolayers were treated with 0·034 mmolar, 0·068 mmolar and 0·136 mmolar BHT for 24 h. Each monolayer was detached from the glass with versene and collected in a test tube, resuspended in suitable known amounts of PBS free from calcium and magnesium ions and counted in a haemocytometer. The percentage inhibition of increase in cell numbers was calculated according to the equation

$$\frac{\text{increase in cell No. (DMSO control)} - \text{increase in cell No. (test)}}{\text{increase in cell No. (DMSO control)}} \times 100 \text{ (ref. 14)}$$

In other experiments, monolayers which had been treated with 0·136 mmolar BHT for 24 h were washed with growth medium and grown for a further 24 h in fresh growth medium. Simultaneously, other cultures were treated for 48 h. DMSO controls were treated in a similar fashion.

The *MK* cell numbers showed an inhibition proportional to the concentration of BHT used (Fig. 1). This 24 h inhibition was reversible within a further 24 h. [illegible] treated for 48 h showed a greater inhibition than those treated for 24 h.

Inhibition of cell numbers may result either from [illegible] death or from a sub-lethal depression of cellular metabolism. *MK* cells were therefore grown on covers[illegible] and treated with 0·136 mmolar BHT for 24 h and [illegible] with May-Grünwald-Giemsa. There was no apparent morphological difference between the BHT-treated [illegible] DMSO control cells when examined by light microscopy.

Three separate series containing test, control [illegible] DMSO control cell cultures were labelled with $^3$H-Tdr, $^3$H-UR or ($^{14}$C)-leucine after treatment with 0·136 [illegible] BHT for 0·5, 3 and 24 h: male *MK* cells were [illegible]. The rate of synthesis of DNA and RNA was also [illegible] in a repeated experiment using female cells after treatment for 0·5, 1, 3, 5 and 7 h.

Fig. 2 shows that the rate of RNA synthesis was [illegible] affected by the BHT, male cells showing 50 per cent inhibition and the female 95 per cent inhibition. In the male cells, protein synthesis seemed to be the [illegible] inhibited, decreasing by only 15 per cent. In both experiments, depression was rapid, and was maximal within 30 min.

A foreign compound may be toxic to an organism in its native form, or as a metabolic intermediate before its excretion[15]. Because the metabolism of BHT in the *MK* cell culture system has not yet been proved. It was [illegible] to be important to test two BHT metabolites for inhibitory effects. Each of three groups of *MK* cell monolayers were treated with either BHT, BHT-acid or BHT-alcohol, at a molarity of 0·136 mmolar. After 1, 3 and 5 h the cells were labelled with $^3$H-UR and the percentage uptake into RNA, compared with DMSO controls, was

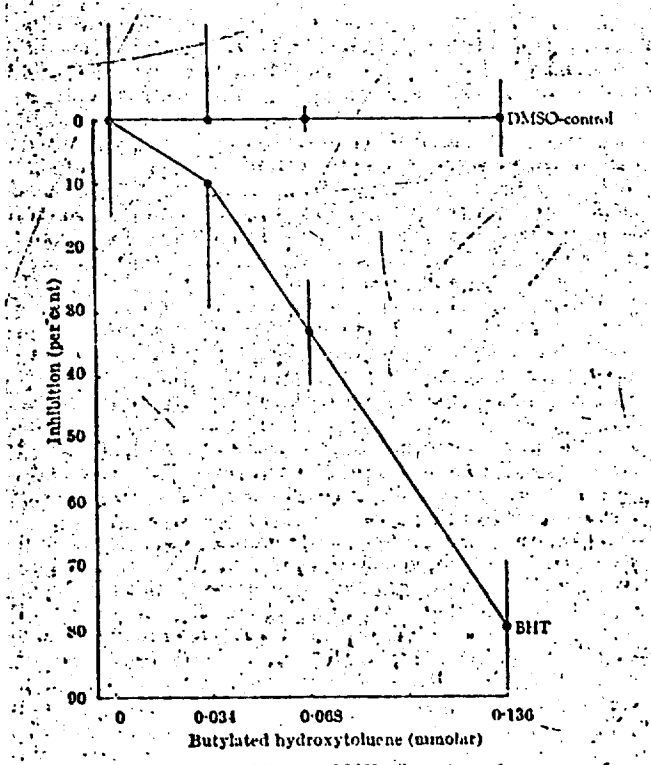


Fig. 1. The percentage inhibition of *MK* cell numbers after exposure for 24 h to 0·034, 0·068 and 0·136 mmolar BHT (dissolved in 0·9 per cent DMSO in growth medium). Cultures treated with 0·9 per cent DMSO were assumed to show no inhibition. The 95 per cent confidence limits are marked.

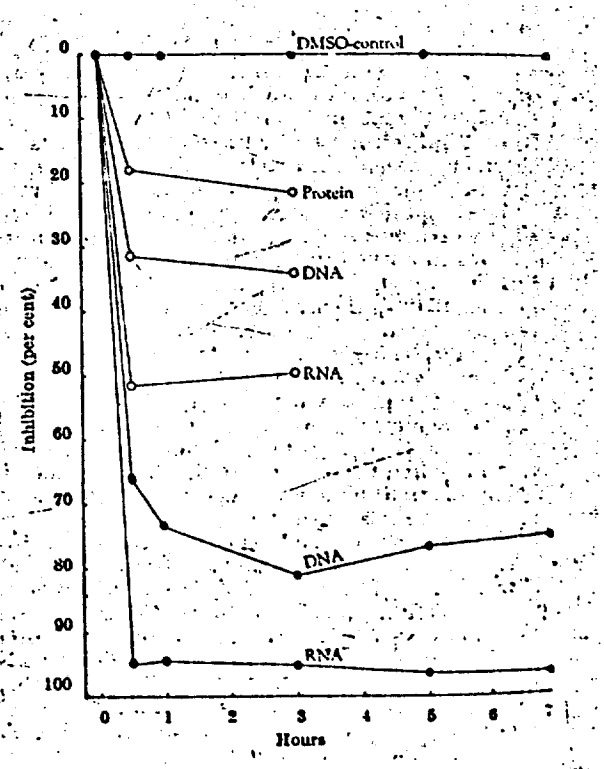


Fig. 2. The percentage inhibition of DNA, RNA and protein synthesis in *MK* cells treated with 0·136 mmolar BHT. All the values [illegible] were significantly lower than those for the DMSO control. ○, male cells; ●, female cells.

calculated. The cells showed little or no significant inhibition of RNA synthesis caused by BHT-acid or BHT-alcohol (Fig. 3).

The possibility that antioxidant activity of BHT induced a metabolic inhibition was tested by comparing the effects of the natural antioxidant α-tocopherol[16,17] and BHT on RNA synthesis. Monolayers of cells were treated with either 0·140 mmolar α-tocopherol or 0·136 mmolar BHT for 0·5, 3 and 24 h, and then labelled with $^3$H-UR. The results in Fig. 4 show that the α-tocopherol had no significant effect on the synthesis of RNA.

Cells were treated for 1 h with 0·136 mmolar BHT. These cells were then washed with prewarmed growth medium, and grown for a further 23 h in fresh growth medium. At 24 h the same cells were again treated with 0·136 mmolar BHT for a further hour and reversed as before. At each reversal, some treated cultures were left in BHT medium to act as BHT controls. The DMSO control group were treated in a way identical to the BHT group of cultures. Cells were labelled with $^3$H-UR after 1, 3, 24, 25 and 26 h. The results in Fig. 5 show that a complete and rapid reversal of RNA inhibition occurred after removal of the BHT. In all experiments, the DMSO controls showed only slight, if any, variation from the control results.

In addition to these experiments, a duplicate set of precooled control cells were labelled at +4° C for 20 min with $^3$H-UR to measure RNA synthesis in cells with a greatly reduced metabolic turnover. These cells showed a 90 per cent inhibition in the synthetic rate when compared with the 37° C controls. A variability in the cellular inhibition caused by 0·136 mmolar BHT was noted in some experiments. This seemed to be correlated with the cell sex; cells from male animals were relatively less affected, and female cells showed a wider range of results. The significant decrease in *MK* cell numbers treated for 24 h with 0·136 mmolar BHT seems to result from a rapid

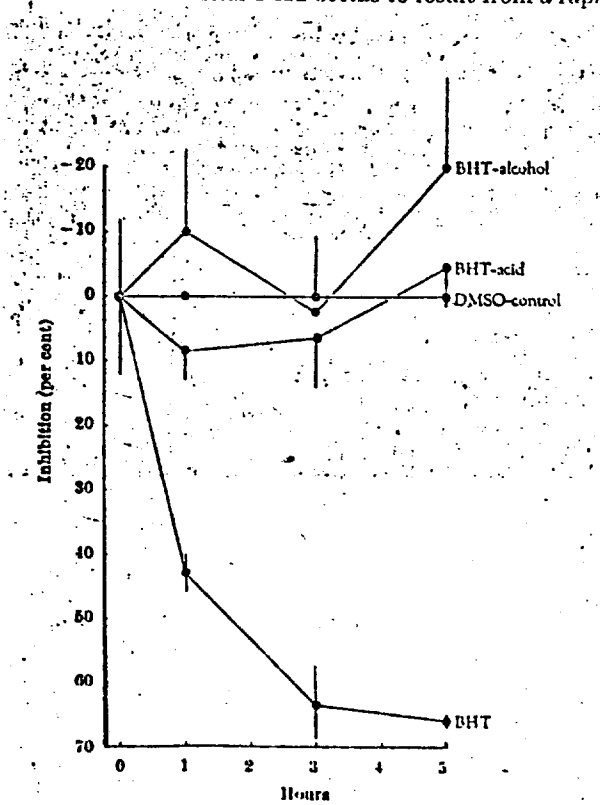


Fig. 3. The percentage inhibition of $^3$H-UR incorporation into *MK* cell RNA caused by 0·136 mmolar BHT and the BHT metabolites 3,5-ditertbutyl-4-hydroxybenzoic acid (BHT-acid), and 3,5-ditertbutyl-4-hydroxybenzyl alcohol (BHT-alcohol). The 95 per cent confidence limits are given, those of the metabolites and the DMSO control all overlapping.

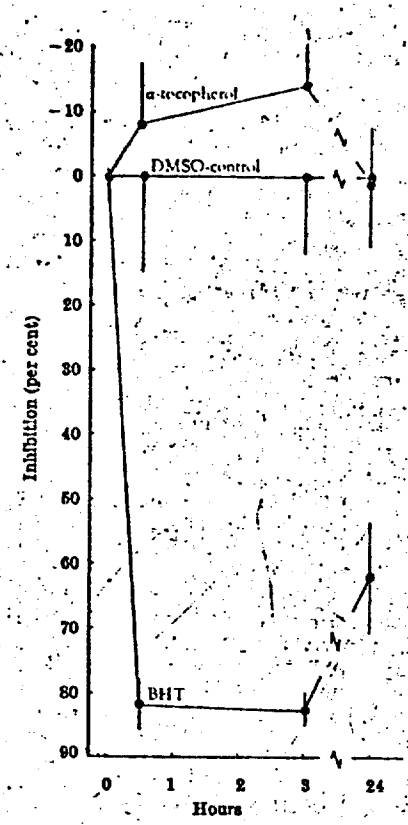


Fig. 4. The percentage inhibition of $^3$H-UR incorporation into *MK* cell RNA caused by 0·136 mmolar BHT and 0·14 mmolar α-tocopherol. The 95 per cent confidence limits are shown.

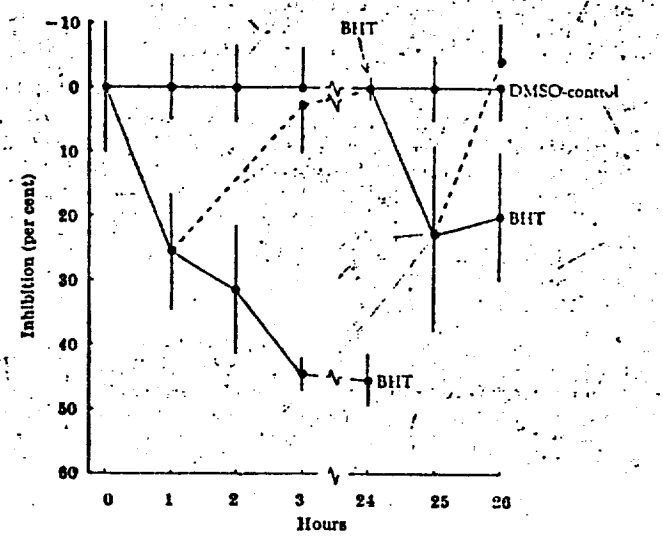


Fig. 5. The reversibility of inhibition of *MK* cell RNA-synthesis after exposure to 0·136 mmolar BHT for 1 h. The reversed cells were re-treated for 1 h with BHT at 24 h, and again reversed. 95 per cent confidence limits are shown. - - - -, Reversed cells in growth medium.

depression of DNA, RNA and protein synthesis which would result in a lowered mitotic index[18].

BHT is a highly lipid soluble compound[19], and thus will associate with the lipids of the plasmalemma and the intracellular membrane systems. If this lipid association were to cause configurational changes[20-22] in biochemically active and/or structural components of the membrane for example, by altering the membrane water structure[23] then it might interfere with the normal metabolic system, situated partially or wholly in these membranes[21-24]. Here it is

important to note that two BHT metabolites, 3,5-ditert-butyl-4-hydroxybenzoic acid and 3,5-ditertbutyl-4-hydroxybenzyl alcohol, both hydrophobic compounds, did not cause inhibition of RNA synthesis at concentrations of 0·136 mmolar. This difference may be the result of a specific characteristic of the molecule-BHT or, alternatively, of a more rapid excretion of the metabolites.

The capacity of various compounds to prevent oxidation has been measured by the AOM, or "active oxygen method"[27]. α-Tocopherol has a recorded AOM value of 83, compared with 67 for BHT and 23 for unprotected controls[2]. In the experiments reported here, however, 0·14 mmolar α-tocopherol does not inhibit RNA synthesis comparable with that produced by 0·136 mmolar BHT, despite its hydrophobic nature and similar antioxidant activity.

The *in vivo* metabolism of BHT has been studied in rats and rabbits[6-8,28]. This occurs chiefly in the liver, involving the enterohepatic circulation[7,28]. The capability of *MK* cells to metabolize BHT *in vitro* has not been proved. In both the cell number and labelling experiments, however, the depressions induced by BHT were rapidly reversed by removal of the BHT, and this may be caused by the *MK* cells converting BHT into non-inhibitory metabolites. A variation in results seemed to be correlated with cell sex; male cells were usually less affected by the BHT than were the female cells. The possibility of BHT being more rapidly metabolized in the male *MK* cells arises. *In vivo*, the hydroxylating activity of the liver in untreated rats is greater in males than females[29]; also Catz and Yaffe[31] have shown that hexobarbital sleeping times in female rats varied with the oestrous cycle.

The concentrations of BHT permitted for use in such foods as butter, lard and margarine is 100–200 p.p.m., and 1,000 p.p.m. is allowed in essential oils[22]. *In vivo* oral doses of 500 mg of BHT/kg body weight cause an increase in the relative liver weight of rats[33]. In these experiments, 0·136 mmolar BHT, or 30 p.p.m., caused an inhibition of cellular metabolism, leading to a relative fall in cell numbers, in $4 \times 10^5$ cells, weighing approximately 46 mg. Although this *in vitro* dose is relatively high, it is difficult to make a direct comparison with the *in vivo* experimental results because of the different effects measured. Also, selective concentration of oral doses of BHT may occur *in vivo*, such as in the small intestinal wall during lipid absorption[34] and in the liver during metabolism.

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# Effect of Coumarin, BHT and Phenobarbitone on Protein Synthesis in the Rat Liver

J. G. NIEVEL

*British Industrial Biological Research Association, Woodmansterne Road, Carshalton, Surrey, England*

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**Abstract**—The capacity of rat-liver microsomes to incorporate labelled amino acids into protein *in vitro* was increased when rats were pretreated with coumarin, BHT or phenobarbitone for up to 7 days. The highest specific activities were obtained with doses of 450 mg coumarin/kg or 450 mg BHT/kg, given orally, or with intraperitoneal injections of 60 mg phenobarbitone/kg. Treatment for 1–3 days did not change the distribution of ribonucleoprotein particles as separated by sucrose density gradient centrifugation. The increased *in vitro* incorporation of amino acids was demonstrable in both microsomal and 'pH 5·0 enzyme' fractions, but was mainly in the former. BHT and phenobarbitone stimulated the *in vivo* incorporation of amino acids, mostly into proteins of the endoplasmic reticulum, whereas in the case of coumarin the effect was more widespread.

## INTRODUCTION

Many compounds produce changes in the rate of hepatic protein synthesis (Smuckler & Barker, 1966). Those which are given in sufficient doses to produce severe toxic effects usually inhibit protein synthesis by interfering with the microsomal incorporation of amino acids (Mizrahi & Emmelot, 1964; Smuckler & Benditt, 1965; Mager, Bornstein & Halbreich, 1965; Richter, 1965; Weksler & Gelboin, 1967). However, when given in low doses over prolonged periods, many compounds possessing very different chemical structures increase this capacity of liver microsomes (Gelboin & Sokoloff, 1964; Kato, Loeb & Gelboin, 1965; Kunz, Schaude, Schimassek, Schmid & Siess, 1966; Nievel & Golberg, 1967; Nievel & Golberg, 1968). This effect, also produced by many carcinogens, occurs concomitantly with metabolic or histopathological signs of cell damage. Substances of low toxicity, such as food additives, may enhance amino-acid incorporation when the only obvious effect is enlargement of what is often a histologically normal liver. One such compound is butylated hydroxytoluene (BHT). In this instance, as in many others, hepatomegaly is associated with an increase in smooth endoplasmic reticulum and increased nucleoli (Lane & Lieber, 1967). Another food additive, coumarin, produces liver enlargement accompanied by histopathological change, and in addition causes histopathological changes in other organs when administered in large doses (Hazleton, Tusing, Zeitlin, Thiessen & Murer, 1956). The effects of these two compounds and their derivatives on cytoplasmic enzymes have been studied by Feuer, Golberg & Le Pelley (1965) and by Gilbert & Golberg (1965) in an attempt to clarify the mechanisms of liver enlargement. Since enzymes are end-products of protein synthesis, their levels of activity constitute only indirect evidence pointing to the initial biochemical

site of inhibition of enzyme synthesis. Coumarin *in vitro* in a 1-mM concentration reduced the rate of hepatic protein synthesis (Couri & Wosilait, 1966), yet *in vivo* this compound increased the capacity of microsomes to incorporate amino acids, an effect sensitive to actinomycin (Nievel & Golberg, 1967; Nievel & Golberg, 1968).

As part of a programme designed to investigate the effects of compounds on protein-synthesizing mechanisms, the present paper extends these observations.

## EXPERIMENTAL

*Materials.* The compounds used were: $^{14}$C-leucine (U), $^{14}$C-L-phenylalanine (U), $^{14}$C-amino-acid mixture of Chlorella hydrolysate and coumarin-T-(9), all from the Radiochemical Centre, Amersham, Bucks; $^{14}$C-BHT from the New England Nuclear Corporation, Boston, Mass., USA; ATP disodium salt, GTP trisodium salt, phosphoenolypyruvate and pyruvate kinase from the Sigma London Chemical Co. Ltd., London; sodium lauryl sulphate and phenobarbitone from British Drug Houses, Ltd., London; scintillators (PPO and POPOP) from Nuclear Enterprises (G.B.) Ltd., Edinburgh; NCS solubilizer from Continental Distributors, Ltd., London; coumarin from Bush Boake Allen, Ltd., London; BHT from Imperial Chemical Industries, Ltd., Alderley Park, Nr. Macclesfield, Cheshire.

*Animals and treatment.* Except where otherwise stated, groups of three or four female Wistar rats (body weight 80–100 g) were given daily doses of coumarin (146 mg/kg) or BHT (220 mg/kg) in solution in arachis oil by intubation, or phenobarbitone (100 mg/kg) by intraperitoneal injection for 1–7 days. Control animals were given arachis oil by intubation. The doses were all administered at the same time of day and, when the animals were subsequently killed, this too was performed at a constant time of day. In another series of experiments, groups of four female Wistar rats received a range of doses of coumarin (15–450 mg/kg/day orally), BHT (15–800 mg/kg/day orally) or phenobarbitone (5–120 mg/kg/day intraperitoneally) for 7 days before sacrifice.

### In vitro *protein synthesis*

*Preparation of liver homogenate fractions.* Rats treated for 3, 5 or 7 days with coumarin, BHT or phenobarbitone were fasted for 12 or 24 hr and then killed by decapitation. The liver from each rat was removed, blotted clean, weighed and homogenized at 0°C in a medium consisting of 0·15 M-sucrose, 0·01 M-$MgCl_2$, 0·025 M-KCl and 0·035 M-tris buffer, pH 7·8. The concentration of the homogenate was always 20% and the homogenization was carried out in a Potter–Elvehjem glass–teflon pestle homogenizer, always under exactly the same conditions, to obtain a random distribution of endoplasmic-reticulum fragments. The homogenate was filtered through three layers of gauze and centrifuged at 4°C for 15 min at 15,000 g in an MSE '18' refrigerated centrifuge. The postmitochondrial supernatant was then further fractionated at 104,000 g for 60 min at 4°C in an MSE Superspeed '50' centrifuge to obtain microsomal preparations. The pellets were washed with the homogenizing medium and the tubes were wiped. The samples were resuspended in the homogenizing medium at a concentration of 6·0 mg protein/ml.

For the preparation of 'pH 5·0 enzymes', the 15,000 g postmitochondrial supernatant was adjusted to pH 5·1 with N-acetic acid and the precipitates were collected, washed with buffer at the same pH and dissolved in the homogenizing medium. A solution of 2–3 mg protein/ml was prepared and all samples of *in vitro* protein synthesis were incubated with an equal amount of the 'pH 5·0 enzyme' protein.

*In vitro incubation.* Microsomes were incubated under conditions in which protein synthesis takes place (Von der Decken & Campbell, 1962; Campbell, Serck-Hanssen & Lowe, 1965). The incubation mixture contained 1–2 $\mu$c of uniformly labelled $^{14}$C-phenylalanine (U) or $^{14}$C-amino-acid mixture of Chlorella hydrolysate, 2–4 $\mu$mole ATP, 0·25 $\mu$mole GTP, 15–30 $\mu$mole phosphoenolypyruvate and 15 $\mu$g pyruvate kinase in homogenizing buffer. The Mg concentration was adjusted to 8 mM, and 0·1 ml 'pH 5·0 enzymes' (0·2–0·3 mg protein) and 0·4 ml microsomal suspension (2·4 mg protein) in a final volume of 1 ml were added to the reaction mixture to achieve optimal incorporation. The incubation was carried out in air at 37°C for 10, 20 and 30 min.

*Determination of radioactivity.* At the end of the incubation, the protein present in the samples was precipitated at 4°C with trichloracetic acid (TCA) containing unlabelled phenylalanine or amino-acid mixture, in an amount to give a final TCA concentration of 10%. The precipitated proteins were centrifuged and washed twice with 5% TCA and extracted at 90°C with 5% TCA for 20 min. The precipitate was subsequently washed again with 5% TCA and then with absolute alcohol. Lipids were extracted by two consecutive treatments with chloroform–methanol (1:2, by vol.) for 20 min each, then with methanol–ether (3:1, by vol.). The pellets were washed successively with methanol and with ether, dried and dissolved in NCS solubilizer. Aliquots were transferred to the counting vials and 10 ml of scintillator consisting of 0·4% PPO and 0·005% POPOP in toluene were added. Samples were counted in a Nuclear Chicago model 720 Liquid Scintillation Spectrometer using the channel ratio method for quenching correction.

### In vivo *protein synthesis*

*Treatment.* After treatment for 1–7 days with coumarin, BHT or phenobarbitone, as described, rats were injected subcutaneously with 2·5 $\mu$c $^{14}$C-L-leucine (U) and 60 min later were killed by decapitation. The livers were quickly excised for homogenization.

*Preparation of liver-homogenate fractions.* The livers were homogenized in ice-cold medium composed of 25 ml 0·01 M-tris buffer, pH 7·6, containing 0·25 M-sucrose, and 0·003 M-$CaCl_2$. In each experiment a 16% homogenate was prepared and filtered through three layers of gauze. Crude nuclear and mitochondrial pellets were prepared by centrifuging the homogenate at 12,500 rev/min for 20 min at 2°C in a Spinco L-4 ultracentrifuge using the type '30' rotor. The nuclei were purified by resuspending the pellets in 0·01 M-tris buffer, pH 7·6, containing 2·2 M-sucrose and 0·002 M-$CaCl_2$, and centrifuging at 22,500 rev/min for 60 min at 2°C. The purified nuclear pellets were finally resuspended in a medium containing 0·01 M-tris buffer, pH 7·6, 0·25 M-sucrose and 0·003 M-$CaCl_2$. The postmitochondrial supernatant was centrifuged at 27,500 rev/min for 90 min at 2°C to obtain the microsomal pellet. The microsomes were resuspended in a medium containing 0·05 M-tris buffer, pH 7·6, 0·025 M-KCl, 0·005 M-$MgCl_2$ and 0·25 M-sucrose. Aliquots were taken from the purified nuclear, microsomal and postmicrosomal supernatant fractions for determination of protein content and radioactivity.

*Determination of radioactivity.* Ammonium hydroxide, was added to each sample to give a final concentration of 1 M. The samples were left standing at room temperature for 2 hr and the proteins were precipitated with TCA at 15% final concentration. The samples were subsequently purified by two extractions with 5% TCA and two with chloroform–methanol (1:2, by vol.) at 40°C for 30 min. The proteins were washed with ethanol and ether, dried, dissolved in NCS solubilizer and mixed with toluene containing scintillator for counting.

*Subcellular distribution of labelled coumarin and BHT*

Rats were treated with coumarin or BHT, as described for the previous experiments, but the test materials contained tritiated coumarin (321 $\mu$c/mmole) or $^{14}$C-BHT (39·5 $\mu$c/kg of specific activity 0·295 $\mu$c/mg). The livers were homogenized in ice-cold tris buffer, pH 7·6, containing 0·25 M-sucrose and 0·003 M-$CaCl_2$, with a Potter–Elvehjem homogenizer to give 10% homogenates, which were centrifuged at 2500 rev/min for 15 min at 2°C in an MSE '18' centrifuge. The nuclei were purified by resuspending the pellets in 0·01 M-tris buffer, pH 7·6, containing 2·2 M-sucrose and 0·0002 M-$CaCl_2$ and centrifuging at 22,500 rev/min for 60 min at 2°C in a Spinco model L-4 ultracentrifuge using the type '30' rotor. Centrifugation of the postnuclear supernatant at 12,500 rev/min for 10 min at 2°C yielded a crude mitochondrial pellet. Microsomes were prepared from the postmitochondrial supernatant by centrifuging at 27,500 rev/min for 90 min at 2°C in the type '30' rotor. The microsomal, mitochondrial and purified nuclear pellets were resuspended in distilled water and from these and from the postmitochondrial supernatants, aliquots were taken and dissolved in 0·02 M-NaOH containing 0·1% sodium lauryl sulphate. Samples were taken for protein determination and aliquots were neutralized, evaporated to dryness, dissolved in 0·5 ml NCS solubilizer and mixed with toluene containing scintillator for counting.

*Separation of the ribonucleoprotein particles on a sucrose density gradient*

The rapidly-excised livers of rats treated with coumarin or BHT, as described, for 3 days were homogenized in a medium containing 0·25 M-sucrose, 0·025 M-KCl, 0·005 M-$MgCl_2$ and 0·05 M-tris buffer, pH 7·6, at 0°C. The 15,000 g postmitochondrial supernatant was treated with 0·9% sodium deoxycholate for 15 min at 0°C and layered on the top of a precooled 14–30% linear sucrose gradient, made up in a buffer containing 0·25 M sucrose, 0·025 M-KCl, 0·005 M-$MgCl_2$ and 0·01 M-tris, pH 7·6. Two ml 50% sucrose was present at the bottom of each centrifuge tube to form a cushion. The ribosomes were sedimented on this gradient by centrifuging the tubes in a Spinco model L-4 refrigerated centrifuge at 65,000 $g_{av.}$ for 3 hr at 2°C using an SW 25·1 rotor. At the end of the centrifugation, 30 fractions were taken from the bottoms of the tubes and diluted with water and the absorption was recorded between 200 and 300 m$\mu$.

*Determination of ATPase activity*

Whole livers from rats treated orally with coumarin (15–450 mg/kg/day) or BHT (15–800 mg/kg/day) or intraperitoneally with phenobarbitone (5–120 mg/kg/day) for 7 days were homogenized in 10 volumes of ice-cold medium containing 0·15 M-sucrose, 0·025 M-KCl, 0·01 M-$MgCl_2$ and 0·036 M-tris buffer, pH 7·8. From the 15,000 g postmitochondrial supernatant, microsomes were prepared by centrifuging the samples at 104,000 g for 60 min in the MSE '50' refrigerated ultracentrifuge. The tubes were wiped and the pellets were resuspended in 0·2 M-histidine buffer, pH 7·5, containing 0·15 M-KCl. The microsomes were incubated in a medium containing ATP for 5–15 min at 28°C (Wayne & Kielley, 1955). The reaction was stopped by adding TCA to give a final concentration of 5% at 0°C and the samples were centrifuged at 2500 rev/min for 10 min. The supernatants were assayed for inorganic phosphate (Martin & Doty, 1949).

## RESULTS

*Effect of test compounds on microsomal protein synthesis* in vitro

Administration of 3–7 daily doses of phenobarbitone, coumarin or BHT to rats increased the subsequent rate of incorporation of phenylalanine or Chlorella hydrolysate amino-acid mixture by liver microsomes *in vitro* (Table 1). No observations were made after 7 days. With all three compounds, the stimulating factor was observed in both the microsomal and 'pH 5·0 enzyme' fractions, but the highest activity was observed using treated microsomes. By day 5, all three compounds had produced an increase of approximately 50% in the rate of microsomal amino-acid incorporation, when combined with 'pH 5·0 enzymes' from either the control or test animals. By day 7, this had increased to 230, 238 and 168% of the control values for coumarin, BHT and phenobarbitone, respectively. This increase was due mainly to amino-acid incorporation by the microsomes.

The effects of different daily doses of coumarin given for 7 days are shown in Table 2. When 15 mg coumarin/kg was given daily for 7 days, only slight increases in amino-acid incorporation by microsomes were observed. At this dosage level, liver weight was not increased; there was a non-significant increase in microsomal protein. Coumarin at 50 mg/kg/day produced significant increases in microsomal protein synthesis and microsomal protein content. These increases continued up to the maximum dose administered (450 mg/kg).

BHT and phenobarbitone showed effects similar to those of coumarin (Table 3). After an initial slight depression of protein synthesis at dose levels of 15 mg BHT/kg and 5 mg phenobarbitone/kg, the rate of amino-acid incorporation in the microsomes of the whole liver significantly increased up to doses of 450 mg BHT/kg or 60 mg phenobarbitone/kg given daily for 7 days. BHT and phenobarbitone also increased the microsomal protein content, which reached maximum values after treatment with 450 mg BHT/kg or 60 mg phenobarbitone/kg treatment. At these dose levels the specific activity of the microsomes, or the rate of incorporation of amino acids into the microsomes, reached its highest values, at 375 and 286% of the control values with BHT and phenobarbitone, respectively. With higher doses of BHT (800 mg/kg) or phenobarbitone (120 mg/kg) the increase in protein synthesis was slightly less marked.

*Effect of test compounds on* in vivo *protein synthesis*

Treatment for 7 days with coumarin, BHT or phenobarbitone increased the *in vivo* incorporation of labelled leucine into liver proteins (Table 4). BHT and phenobarbitone exerted their maximal effect on microsomal protein synthesis, whereas coumarin affected both the microsomal and soluble fractions.

*Subcellular distribution of coumarin and BHT*

Table 5 shows the changes in subcellular distribution of $^{3}$H-coumarin and $^{14}$C-BHT between various cell fractions. The initial distribution of radioactivity after administration of coumarin was mainly in the microsomal and supernatant fractions. After 5 days, the nuclear fraction and, to a lesser degree, the mitochondrial fraction, showed an accumulation at the expense of the supernatant fraction. Radioactivity derived from $^{14}$C-BHT was also present mainly in the supernatant and microsomal fractions. After 5 days, activity in the nuclear fraction had increased threefold with a concomitant fall in the microsomal and supernatant fractions.

Table 1. *Effect of coumarin, BHT and phenobarbitone on protein synthesis by rat-liver microsomes* in vitro

| Treatment | Source of 'pH 5·0 enzyme' (test group) | Rate of amino-acid incorporation after treatment for 3 Days: dpm/mg* | 3 Days: % of control | 5 Days: dpm/mg* | 5 Days: % of control | 7 Days: dpm/mg* | 7 Days: % of control |
|---|---|---|---|---|---|---|---|
| Arachis oil (control) | Control | 11,825 (11,716–11,902) | — | 34,902 (33,748–36,447) | — | 1193 (1061–1260) | — |
| Coumarin (146 mg/kg/day) | Control | 15,830 (15,399–16,261) | 134 | 54,792 (52,576–57,007) | 157 | 2282 (2164–2400) | 191 |
| | Coumarin-treated | 13,593 (13,308–13,928) | 115 | 56,419 (55,033–57,460) | 162 | 2741 (2636–2814) | 230 |
| BHT (220 mg/kg/day) | Control | 15,910 (15,296–16,523) | 135 | 52,829 (50,441–55,216) | 151 | 2456 (2427–2487) | 206 |
| | BHT-treated | 17,330 (16,118–17,988) | 147 | 56,196 (52,173–59,113) | 161 | 2836 (2797–2879) | 238 |
| Phenobarbitone (100 mg/kg/day) | Control | 15,252 (14,897–15,607) | 129 | 54,374 (44,596–46,151) | 130 | 1494 (1458–1529) | 125 |
| | Phenobarbitone-treated | 18,088 (16,510–20,531) | 153 | 54,861 (52,020–56,352) | 157 | 2007 (1770–2373) | 168 |

* mg Microsomal protein.

Values are the means (with ranges and percentages of control values) for groups of three rats given arachis oil (control), coumarin or BHT orally or phenobarbitone intraperitoneally for up to 7 days. Microsomal pellet from control and treated animals was incubated in an appropriate medium with radioactive amino-acid mixture and with 'pH 5·0 enzymes' derived either from controls or from animals treated with the test compound in question. The different specific activities of the radioactive amino acids used at 3, 5 and 7 days is reflected in the wide variation in the results obtained with the control groups.

Table 2. *Effect of seven daily oral doses of coumarin on protein synthesis by rat-liver microsomes*

| Treatment | Final liver wt (g) | Microsomal protein (mg/g liver) | Microsomal protein (mg/total liver wt) | Microsomal protein (mg/rm)† | Activity (dpm/mg microsomal protein) | Activity (dpm × $10^{-3}$/total microsomal protein) |
|---|---|---|---|---|---|---|
| Control | 4·11 ± 0·77 | 20·1 ± 2·9 (100) | 82·6 ± 12·4 (100) | 2·21 ± 0·06 | 14,230 ± 1800 (100) | 1191·36 ± 339·7 (100) |
| Coumarin (15 mg/kg/day) | 3·99 ± 0·35 | 23·2 ± 2·7 (115) | 92·3 ± 10·2 (112) | 2·24 ± 0·07 | 16,670 ± 2820 (117) | 1530·28 ± 120·6 (128) |
| Control | 3·56 ± 0·13 | 39·1 ± 2·2 (100) | 139·1 ± 6·0 (100) | 1·936 ± 0·04 | 11,644 ± 80 (100) | 1619·64 ± 82·6 (100) |
| Coumarin (15 mg/kg/day) | 3·29 ± 0·11* | 41·5 ± 3·9 (106) | 136·3 ± 13·6 (98) | 1·91 ± 0·03 | 13,030 ± 1150 (112) | 1772·54 ± 359·1 (109) |
| Control | 3·42 ± 0·31 | 18·5 ± 1·9 (100) | 62·7 ± 4·3 (100) | 1·875 ± 0·19 | 10,203 ± 2070 (100) | 638·25 ± 75·6 (100) |
| Coumarin (50 mg/kg/day) | 3·35 ± 0·16 | 20·3 ± 2·4 (110) | 67·7 ± 5·7 (108) | 1·984 ± 0·12 | 10,334 ± 2120 (101) | 695·84 ± 124·0 (109) |
| Control | 3·58 ± 0·36 | 27·9 ± 3·8 (100) | 98·4 ± 5·4 (100) | 1·76 ± 0·04 | 11,180 ± 430 (100) | 1130·46 ± 44·9 (100) |
| Coumarin (50 mg/kg/day) | 3·71 ± 0·18 | 34·7 ± 2·2 (124)* | 128·3 ± 5·8 (130)*** | 1·733 ± 0·03 | 12,790 ± 460 (114)** | 1642·70 ± 134·3 (145)** |
| Control | 3·43 ± 0·33 | 23·5 ± 2·3 (100) | 80·7 ± 12·0 (100) | 2·59 ± 0·16 | 7239 ± 720 (100) | 580·62 ± 132·3 (100) |
| Coumarin (150 mg/kg/day) | 3·94 ± 0·33 | 28·3 ± 2·5 (120)* | 111·0 ± 13·2 (138)** | 2·540 ± 0·22 | 13,391 ± 400 (171)*** | 1374·65 ± 117·3 (237)*** |
| Control | 3·97 ± 0·38 | 26·9 ± 2·1 (100) | 106·4 ± 9·9 (100) | 1·891 ± 0·13 | 9429 ± 1080 (100) | 1008·16 ± 323·1 (100) |
| Coumarin (150 mg/kg/day) | 4·39 ± 0·28 | 32·7 ± 2·8 (122)* | 143·0 ± 9·9 (134)** | 1·798 ± 0·08 | 14,045 ± 2286 (149)* | 1997·68 ± 194·6 (198)*** |
| Control | 3·77 ± 0·25 | 20·4 ± 1·2 (100) | 77·2 ± 9·2 (100) | 2·218 ± 0·02 | 7955 ± 570 (100) | 612·31 ± 124·0 (100) |
| Coumarin (450 mg/kg/day) | 4·43 ± 0·39* | 31·4 ± 4·2 (154)*** | 138·2 ± 8·1 (179)*** | 2·125 ± 0·02** | 12,042 ± 1200 (151)*** | 1657·54 ± 86·2 (271)*** |
| Control | 3·98 ± 0·12 | 29·9 ± 2·2 (100) | 118·8 ± 7·7 (100) | 1·890 ± 0·93 | 8220 ± 490 (100) | 978·27 ± 98·9 (100) |
| Coumarin (450 mg/kg/day) | 4·92 ± 0·26** | 43·9 ± 0·9 (147)** | 215·8 ± 6·8 (182)*** | 1·740 | 13,692 ± 970 (167)** | 2947·33 ± 114·8 (301)*** |

† mg/rm = mg microsomal protein added to the reaction mixture for *in vitro* incorporation.
Values are means ± SD for groups of four animals and those marked with asterisks differ significantly (Student's *t* test) from control values: *$P < 0·05$; **$P < 0·01$; ***$P < 0·001$.

Table 3. *Effect of seven daily doses of BHT (given orally) or phenobarbitone (given intraperitoneally) on protein synthesis by rat-liver microsomes*

| Treatment | Final liver wt (g) | Microsomal protein (mg/g liver) | Microsomal protein (mg/total liver wt) | Microsomal protein (mg/rm)† | Activity (dpm/mg microsomal protein) | Activity (dpm × $10^{-3}$/total microsomal protein) |
|---|---|---|---|---|---|---|
| Control | 4·27 ± 0·29 | 24·8 ± 1·1 (100) | 105·9 ± 13·3 (100) | 1·730 ± 0·07 | 14,738 ± 1736 (100) | 1571·15 ± 327·6 (100) |
| BHT (15 mg/kg/day) | 3·36 ± 0·10** | 27·4 ± 2·7 (110) | 92·0 ± 11·4 (87) | 1·735 ± 0·07 | 9480 ± 723 (64)** | 876·26 ± 167·08 (56)* |
| Phenobarbitone (5 mg/kg/day) | 3·50 ± 0·24* | 28·3 ± 2·0 (114)* | 99·12 ± 10·1 (94) | 1·732 ± 0·08 | 11,568 ± 2468 (79) | 1143·48 ± 238·75 (73) |
| Control | 4·30 ± 0·38 | 26·0 ± 1·4 (100) | 111·6 ± 9·0 (100) | 1·908 ± 0·07 | 3822 ± 410 (100) | 427·81 ± 70·56 (100) |
| BHT (50 mg/kg/day) | 4·68 ± 0·48 | 28·0 ± 1·9 (108) | 130·6 ± 14·5 (117) | 1·885 ± 0·07 | 5066 ± 960 (133) | 660·04 ± 136·57 (154)* |
| Phenobarbitone (15/mg/kg/day) | 4·87 ± 0·37 | 33·0 ± 2·8 (127)** | 157·6 ± 11·5 (141)*** | 1·796 ± 0·10 | 4924 ± 550 (129)* | 774·63 ± 87·20 (181)*** |
| Control | 3·79 ± 0·10 | 29·2 ± 2·1 (100) | 111·0 ± 10·6 (100) | 1·911 ± 0·101 | 3909 ± 362 (100) | 432·53 ± 38·94 (100) |
| BHT (150 mg/kg/day) | 5·03 ± 0·41** | 34·3 ± 2·1 (117)* | 172·3 ± 16·4 (155)** | 2·047 ± 0·240 | 5109 ± 753 (131)* | 873·91 ± 87·07 (202)*** |
| Phenobarbitone (30 mg/kg/day) | 4·73 ± 0·41* | 34·0 ± 5·9 (116)** | 159·6 ± 24·6 (144)* | 1·866 ± 0·085 | 6981 ± 1291 (179)* | 1099·92 ± 156·90 (254)*** |
| Control | 3·43 ± 0·13 | 26·6 ± 1·5 (100) | 91·5 ± 6·9 (100) | 1·887 ± 0·055 | 3738 ± 431 (100) | 333·88 ± 47·9 (100) |
| BHT (450 mg/kg/day) | 5·26 ± 0·35*** | 41·4 ± 3·2 (156)* | 217·2 ± 11·9 (237)*** | 1·923 ± 0·092 | 5787 ± 560 (155)*** | 1253·68 ± 98·2 (375)*** |
| Phenobarbitone (60 mg/kg/day) | 4·18 ± 0·37** | 46·8 ± 1·7 (176)*** | 195·3 ± 10·8 (213)*** | 2·011 ± 0·133 | 4773 ± 880 (128) | 954·84 ± 216·7 (286)** |
| Control | 3·62 ± 0·09 | 32·0 ± 2·3 (100) | 115·9 ± 8·4 (100) | 2·017 ± 0·056 | 4405 ± 335 (100) | 509·31 ± 345·90 (100) |
| BHT (800 mg/kg/day) | 6·18 ± 0·72** | 39·5 ± 7·9 (123) | 240·4 ± 24·7 (207)** | 2·005 ± 0·120 | 6591 ± 1819 (150) | 1612·32 ± 565·15 (317)* |
| Phenobarbitone (120 mg/kg/day | 4·21 ± 1·27** | 49·7 ± 2·0 (155)*** | 208·9 ± 1·4 (180)*** | 2·126 ± 0·082 | 5488 ± 653 (125) | 1146·05 ± 132·40 (225)*** |

† mg/rm = mg microsomal protein added to the reaction mixture for *in vitro* incorporation.
Values are means ± SD for groups of four animals and those marked with asterisks differ significantly (Student's *t* test) from control values: *$P < 0·05$; **$P < 0·01$; ***$P < 0·001$.

Table 4. *Effect on liver-protein synthesis in vivo of seven daily doses of coumarin or BHT given orally or phenobarbitone given intraperitoneally*

| Treatment | Incorporation of $^{14}$C-leucine (% of controls) in liver-protein fractions | | |
|---|---|---|---|
| | Nuclear | Microsomal | Soluble |
| Arachis oil (control) | 100 | 100 | 100 |
| Coumarin (146 mg/kg/day) | 104 | 130 | 139 |
| BHT (220 mg/kg/day) | 107 | 141 | 110 |
| Phenobarbitone (100 mg/kg/day) | 97 | 110 | 90 |

Values are calculated from results (in dpm/mg protein) obtained in groups of four rats.

Table 5. *Changes in subcellular distribution of radioactivity derived from $^{3}$H-coumarin and $^{14}$C-BHT in liver-cell fractions during oral administration of labelled compounds for up to 5 days*

| Treatment | No. of daily doses | Time after first dose (hr) | Radioactivity of cell fractions (% of total cell radioactivity) | | | |
|---|---|---|---|---|---|---|
| | | | Nuclei | Mitochondria | Microsomes | Supernatant |
| Coumarin (146 mg/kg/day) containing $^{3}$H-coumarin | 1 | 8·25 | 11<br>12 | 17<br>17 | 38<br>36 | 34<br>35 |
| | 1 | 17·25 | 15<br>13 | 22<br>21 | 32<br>36 | 31<br>31 |
| | 3 | 63 | 13<br>20 | 21<br>20 | 43<br>37 | 23<br>23 |
| | 5 | 120 | 21<br>21 | 22<br>27 | 36<br>30 | 21<br>22 |
| BHT (220 mg/kg/day) containing $^{14}$C-BHT | 1 | 8·25 | 7<br>6 | 20<br>21 | 38<br>39 | 35<br>34 |
| | 1 | 17·25 | 8<br>7 | 26<br>24 | 27<br>29 | 39<br>40 |
| | 3 | 63 | 12<br>16 | 26<br>25 | 39<br>36 | 23<br>23 |
| | 5 | 120 | 25<br>19 | 22<br>19 | 31<br>32 | 23<br>30 |

Individual results are given for pairs of samples.

*Effect on distribution of ribonucleoprotein aggregates*

Neither coumarin nor BHT, after three consecutive daily doses of 146 mg/kg and 220 mg/kg respectively, produced changes in the proportion of single particles to ribonucleoprotein aggregates as determined by sedimentation (Fig. 1).

*Effects on ATPase activity*

With increasing dosage, all three compounds produced a decrease in microsomal concentration of ATPase, which was statistically significant at the higher dose levels (Table 6). The only exception to this general finding was an increase following the lowest dose of phenobarbitone. However, the total amount of microsomal protein in the liver increased, and thus

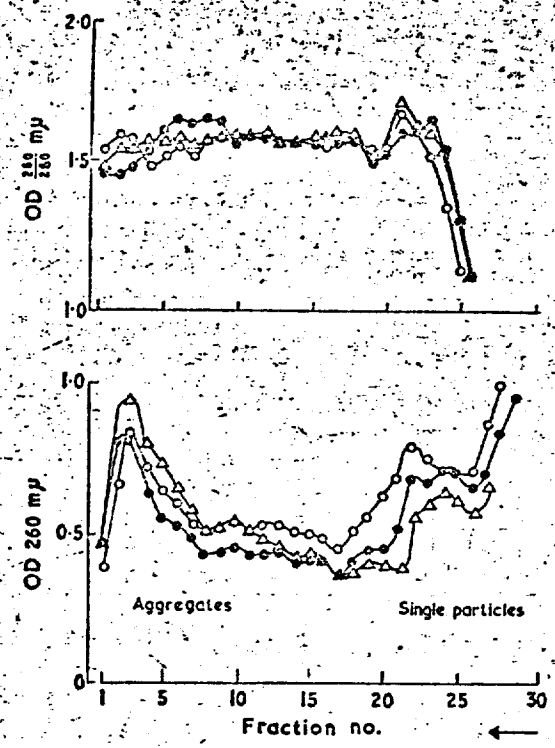


FIG. 1. Effect of three daily oral doses of coumarin (146 mg/kg/day; ○) or BHT (220 mg/kg/day; △) on the distribution of ribonucleoprotein particles of rat liver, as determined by sucrose density gradient centrifugation. The arrow indicates the direction of sedimentation, and ○ the values for control rats.

the changes in microsomal ATPase did not reflect changes in total liver ATPase. Coumarin slightly lowered the total amount of ATPase in liver, but BHT produced a significant increase in total liver ATPase in the group given the lowest dose, and phenobarbitone increased total liver ATPase significantly in doses of 5, 60 and 120 mg/kg/day.

## DISCUSSION

Both BHT and phenobarbitone are inducers of liver microsomal enzymes (Remmer, 1962; Conney & Burns, 1963; Gilbert & Golberg, 1965) but, although it is also lipid-soluble, coumarin does not show a similar effect (Feuer, Gaunt, Golberg & Fairweather, 1965). The morphological changes produced by coumarin are also different from those due to phenobarbitone and BHT; coumarin in high doses brings about histopathological changes while the other two compounds produce hepatomegaly with no histopathological abnormality and, ultrastructurally, a proliferation of smooth endoplasmic reticulum. A further difference is the finding that, while phenobarbitone and BHT stimulate protein synthesis mainly in the microsomal fraction of liver, this effect is spread throughout the cytoplasm in the case of coumarin.

All three compounds were similar in so far as stimulation of protein synthesis was dose-related and relatively long-lasting. There was considerable variation, however, between related results obtained in different studies, and some seasonal variation in results has been

Table 6. *Effect of coumarin, BHT and phenobarbitone on the activity of microsomal ATPase of the rat liver*

| Treatment | $P_i$ (μg/mg microsomal protein) | $P_i$ (μg/total microsomal protein) |
|---|---|---|
| Control | 109 ± 7·11 | 1521 ± 114·27 |
| Coumarin (15 mg/kg/day) | 103·25 ± 11·36 | 1402·5 ± 166·72 |
| Control | 129 ± 11·30 | 1274·5 ± 205·80 |
| Coumarin (50 mg/kg/day) | 99 ± 6·56* | 1268 ± 36·91 |
| Control | 128 ± 8·04 | 1516 ± 141·24 |
| Coumarin (450 mg/kg/day) | 58 ± 3·00*** | 1261 ± 144·25 |
| Control | 88 ± 5·52 | 933 ± 60·50 |
| BHT (15 mg/kg/day) | 150 ± 14·9 | 1368 ± 93·94*** |
| Phenobarbitone (5 mg/kg/day) | 133 ± 12·69** | 1313 ± 106·50* |
| Control | 120·5 ± 24·45 | 1346 ± 323·01 |
| BHT (50 mg/kg/day) | 109 ± 9·33 | 1416 ± 168·40 |
| Phenobarbitone (15 mg/kg/day) | 93 ± 18·95 | 1473 ± 349·65 |
| Control | 170 ± 18·14 | 1552 ± 197·16 |
| BHT (450 mg/kg/day) | 78·5 ± 10·49*** | 1701 ± 246·06 |
| Phenobarbitone (60 mg/kg/day) | 96 ± 5·57*** | 1872 ± 114·72* |
| Control | 141 ± 3·46 | 1630 ± 164·48 |
| BHT (800 mg/kg/day) | 77 ± 28·02* | 1832 ± 406·2 |
| Phenobarbitone (120 mg/kg/day) | 100·5 ± 14·53** | 2094 ± 266·2* |

Values are means ± SD for groups of four rats treated orally with coumarin or BHT or intraperitoneally with phenobarbitone for 7 days. Those marked with asterisks differ significantly (Student's *t* test) from control values: $*P < 0·05$; $**P < 0·01$; $***P < 0·001$.

observed. For this reason some experiments were repeated at different periods, control values were determined separately for each test, and within each test all possible steps were taken to standardize the experimental conditions (such as the taking of all samples at the same time of day). It is likely that changes in the capacity of the microsomes to incorporate labelled amino acids result from simultaneous subjection to both inhibitory and stimulatory effects. Only inhibitory effects are observed when the test compounds are given at low doses, but with higher doses, changes occur at whole-body level which overcome the direct effects of the compound on the actual incorporating system.

The effect on amino-acid incorporation was observed regardless of whether 'pH 5·0 enzymes' were prepared from control or treated animals. Moreover, administration of seven daily doses of 50–450 mg coumarin/kg, 450–800 mg BHT/kg, or 60–120 mg phenobarbitone/kg depressed the microsomal concentration of ATPase but did not reduce the total liver ATPase content. It is unlikely, therefore, that reduced breakdown of ATP or changes in ATP production are the sole explanation for the increased incorporation of amino acids (Hamburger, Halbreich & Mager, 1967).

The possibility that the coumarin effect may be mediated through the adrenal cortex is suggested by the finding that coumarin-stimulated protein synthesis was diminished in adrenalectomized animals (Nievel, 1969a). The long-lasting nature of the effect of coumarin is not in keeping with this suggestion, however, since stimulation of protein synthesis by factors provoking a response of the pituitary–adrenal axis was of short duration (Majumdar, Tsugada & Lieberman, 1967).

Coumarin and BHT absorbed from the gut appear to be distributed throughout the cytoplasmic structures. Both compounds or their metabolic products accumulate in various tissues (Hazleton *et al.* 1956). An increased level of radioactivity due to administration of labelled coumarin or BHT detected in lipoprotein structures 2–3 days after oral intubation may be the cause of the relatively long-lasting effect of these compounds on protein synthesis. The presence of products derived from coumarin and BHT in the nuclei and membrane preparations may affect the protein synthesis in the cytoplasm either by a direct action on ribosomes or indirectly by acting on RNA synthesis. Pretreatment of rats with actinomycin D prevented the initial increase in the rate of microsomal protein synthesis otherwise associated with coumarin treatment (Nievel & Golberg, 1968). However, the effect of actinomycin D is difficult to interpret owing to its complex action when given *in vivo* and the stability of messenger RNA in the isolated liver (Nievel & Dawson, 1967). Hence any suggestion that the primary effect is on RNA synthesis can be no more than speculative. Preliminary work using conditions which only just produced an increase in protein synthesis, did not reveal any enhanced incorporation of labelled precursors into nucleic acids (Nievel, 1969b). It is possible, however, that during induction of microsomal protein synthesis, changes took place in the pool sizes of the nucleic-acid precursors.

Sudden changes in messenger RNA synthesis during protein or enzyme induction are usually reflected in an increase of heavy aggregates. However, no such effect was observed after 3 days' treatment with either coumarin or BHT. The constant proportion of ribosomes could mean either that the initial effect had already been compensated and ribosome formation had increased simultaneously with messenger RNA, or that the ribosomal protein synthesizing machinery was functioning at higher efficiency without any change in the rate of formation of either of its components. A wide variety of compounds, including hormones, has been shown to affect translational control in mammalian tissue in a similar way (Smuckler & Barker, 1966). The present data are insufficient, however, to determine how widely the effect of the three compounds investigated is extended over the protein-synthesizing system from the nucleus to the cytoplasm. Until more precise sites of action of the compounds are delineated (transcription, translation or changes in energy supply or precursors for nucleic-acid synthesis) the present findings cannot be fully interpreted in terms of toxicity.

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## Effet de la coumarine, du HTB et du phénobarbital sur la protéosynthèse dans le foie du rat

Résumé—L'aptitude des microsomes du foie du rat à incorporer *in vitro* aux protéines des acides aminés marqués augmente quand les animaux ont été traités au préalable, pendant jusqu'à 7 jours, à la coumarine, à l'hydroxytoluène butylé (HTB) ou au phénobarbital. L'activité spécifique la plus élevée a été obtenue avec des doses orales de 450 mg de coumarine ou de HTB par kg de poids vif et par des injections intrapéritonéales de 60 mg de phénobarbital par kg de poids vif. Un traitement de 1–3 jours n'a pas modifié la distribution des particules de ribonucléoprotéine séparées par centrifugation dans un gradient de densité de saccharose. L'augmentation d'incorporation *in vitro* d'acides aminés a pu être démontrée aussi bien dans les fractions microsomiques que dans les fractions 'd'enzyme à pH 5,0', mais se manifestait surtout chez les premières. Le HTB et le phénobarbital ont stimulé l'incorporation *in vivo* des acides aminés, principalement dans les protéines du réticulum endoplasmatique; cet effet était plus étendu dans le cas de la coumarine.

## Einfluss von Cumarin, BHT und Phenobarbital auf die Proteinsynthese in der Rattenleber

Zusammenfassung—Die Fähigkeit von Rattenlebermikrosomen, markierte Aminosäuren *in vitro* in Protein einzubauen, war erhöht, wenn die Ratten bis zu 7 Tage lang mit Cumarin, BHT oder Phenobarbital vorbehandelt waren. Die höchste spezifische Aktivität wurde mit oralen Dosen von 450 mg Cumarin/kg oder 450 mg BHT/kg oder aber mit intraperitonealen Injektionen von 60 mg Phenobarbital/kg erhalten. Eine 1–3 Tage währende Behandlung änderte nicht die Verteilung der durch Sucrosedichten-Gradienten bei der Zentrifugierung getrennten Ribonucleoproteinteilchen. Der erhöhte Einbau der Aminosäuren *in vitro* war in mikrosomalen und pH 5,0-Enzymfraktionen nachweisbar, in den ersteren jedoch deutlicher. BHT und Phenobarbital regten den Einbau von Aminosäuren *in vivo* an, vor allem in Proteine des endoplasmischen Reticulum, während im Fall des Cumarin die Wirkung verbreiteter war.

# Application of Thin-Layer Chromatography to the Quantitative Estimation of Antioxidants: BHA, BHT, PG, and NDGA

By MADHU R. SAHASRABUDHE (Food and Drug Directorate, Tunney's Pasture, Ottawa, Ontario, Canada)

**A procedure is described for the quantitative estimation of BHA, BHT, PG, and NDGA in lard. The antioxidants are extracted from n-hexane solutions of the fat with 80% ethyl alcohol and acetonitrile, separated by two-dimensional thin-layer chromatography using benzene and acetonitrile, and estimated quantitatively by colorimetric methods after extraction of the spots. A special flask for concentration and a suction tube assembly for spot pickup are described. Recoveries of the antioxidants from lard were in the following ranges: BHA, 98–101%; BHT, 96–101%; PG, 99–101%; NDGA, 82–93%. The advantages of the method over others in use are that (1) all four antioxidants can be estimated in one aliquot of fat, (2) PG and NDGA can be estimated when present together in mixtures, and (3) the isomers of BHA can be effectively separated.**

Butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG), and nordihydroguaiaretic acid (NDGA) are the four antioxidants most commonly used in foods. Anglin, Mahon, and Chapman (1) described methods which have been widely used for quantitative estimation of the above antioxidants in mixtures. In their method, PG and NDGA were extracted with 72% ethyl alcohol from fat dissolved in petroleum ether or carbon tetrachloride, while BHA and BHT were steam distilled. The only limitation of the method was that PG and NDGA could not be estimated in mixtures.

Lundberg and Halverson (2) reported that a number of antioxidants including BHA, PG, and NDGA could be quantitatively recovered from fat by extraction with 80% ethyl alcohol. Mahon and Chapman found 72% alcohol more suitable for extraction of BHA, PG, and NDGA since it excludes tocopherol. Under these conditions BHT is only partially extracted. Our preliminary experiments indicated that acetonitrile effectively removed BHT from glyceride oil in n-hexane solutions.

Qualitative separation of the antioxidants by thin-layer chromatography (TLC) has been used successfully for pure antioxidant mixtures (4, 5). However, lipid materials associated with antioxidants interfere in the determination. Quantitative estimation by TLC depends upon (1) effective extraction of the antioxidants from the fat, (2) separation of the antioxidants by TLC, and (3) quantitative recovery from TLC plates.

The present paper describes a method for determination of BHA, BHT, propyl gallate, and NDGA in all combinations.

## METHODS

### *Apparatus*

As TLC requires concentrated solutions for effective application, it is desirable to concentrate the solvent. A special evaporation flask was devised for the purpose (Fig. 1 A).

For the recovery of the antioxidants from TLC plates a suction tube assembly was devised (Fig. 1 B).

### *Reagents*

(a) *Silica Gel-G.*—(According to Stahl)

(b) *Benzene.*—Reagent grade.

(c) *Acetonitrile.*—Freshly distilled.

(d) *2,6-Dichloroquinonechloroimide* (CQC) 0.1% in absolute ethyl alcohol spray reagent.

(e) *Ethyl alcohol.*—Distill commercial absolute alcohol in an all-glass apparatus with 0.1% KOH and 0.1% $KMnO_4$. Take the distillate as 100% and dilute to 80%, 50%, and 20% (v/v).

(f) *Ferrous sulfate.*—0.01% $FeSO_4.7H_2O$ in distilled water. (Prepare daily.)

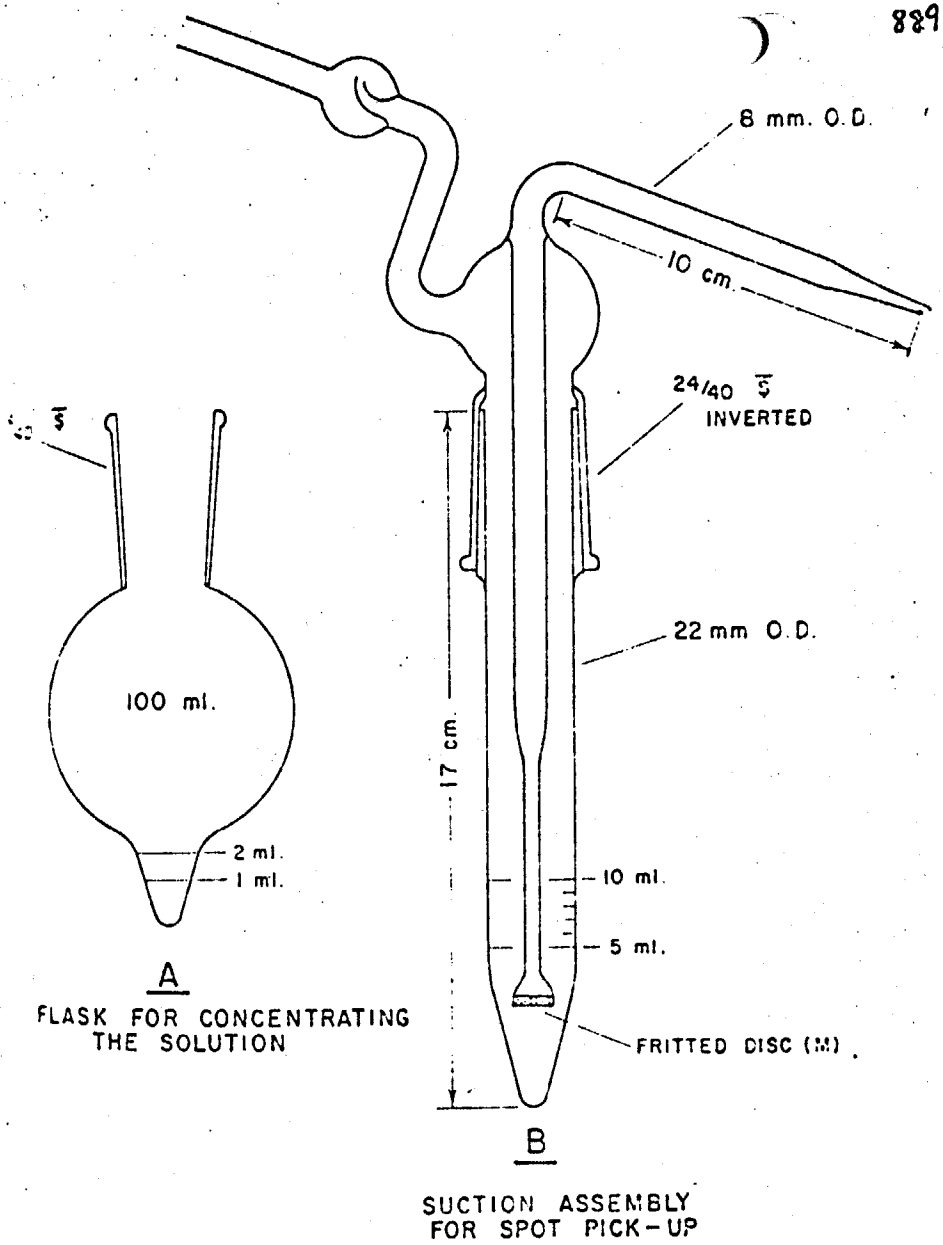


FIG. 1.—A, evaporation flask for concentration of the antioxidant solutions. B, suction assembly for spot pickup.

(g) *Ferric chloride.*—0.2% $FeCl_3.6H_2O$ in distilled water.

(h) *Sodium carbonate-bicarbonate buffer.*—Anhydrous sodium carbonate and 4.2% sodium bicarbonate in distilled water.

(i) *2,2'-Bipyridine reagent (BiP).*—Dissolve 200 mg 2,2'-bipyridine in 1 ml ethyl alcohol and dilute to 100 ml with distilled water. If 2,2'-bipyridine is discolored and sticky, it should be purified as described by Mahon and Chapman (3).

(j) *2,6-Dichloroquinonechloroimide (CQC) reagent.*—Dissolve 10 mg CQC in 100 ml ethyl alcohol. Prepare fresh daily.

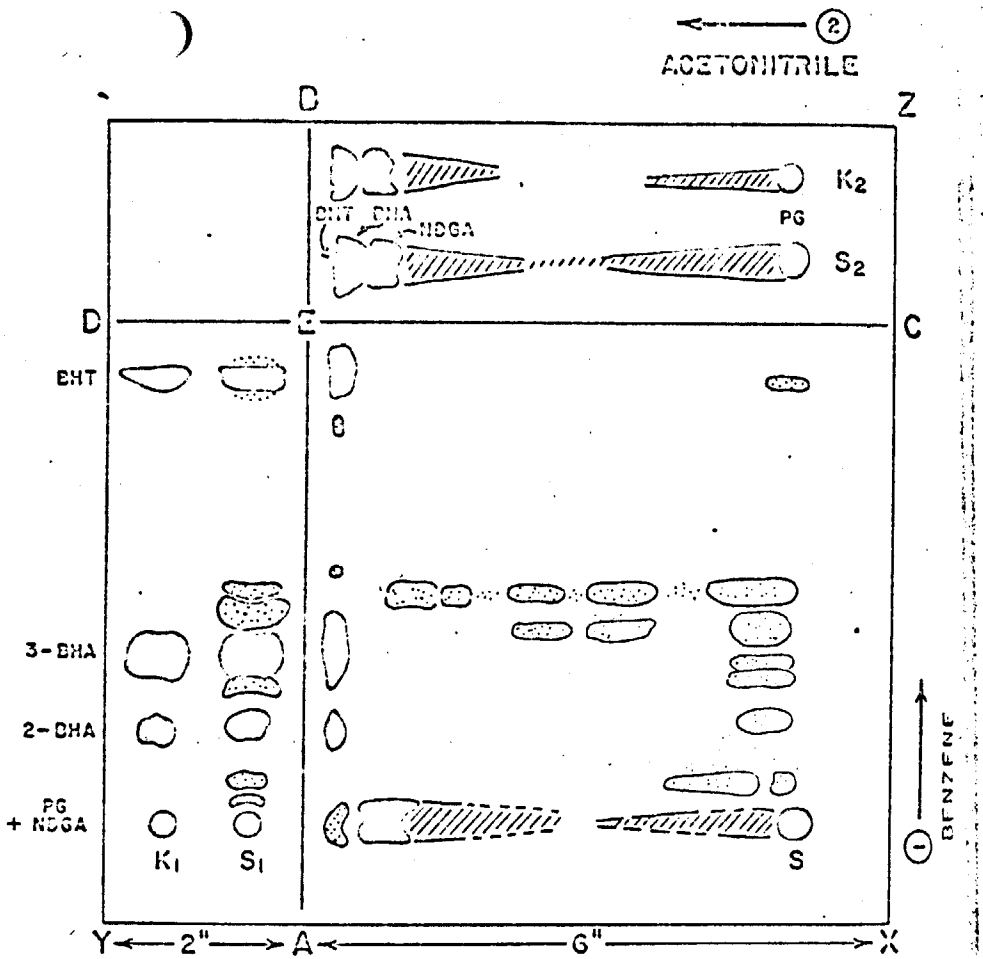


Fig. 2—Diagram of a TLC plate drawn from an actual tracing. Solid black spots are the antioxidants. Streaks obtained with PG and NDGA are shown as shaded areas. Dotted spots and areas represent lipid material. These spots become visible in iodine vapor. In the method, BHA and BHT spots in the area XAEC are not visible. On spraying with CQC the antioxidants are seen as different colored spots, viz., BHA, blue; BHT, yellow; NDGA, purple; and PG, greyish purple.

(k) *Borax buffer.*—2% $Na_2B_4O_7.10\ H_2O$ in distilled water.

(l) *Ammonium acetate.*—2.5% in distilled water.

### Determination

*Extraction of antioxidants.*—Dissolve 10 g lard in 100 ml *n*-hexane in a separatory funnel; extract with four 25 ml portions of 80% ethyl alcohol by inverting the funnel 5 times for each extraction. Then, extract with eight 25 ml portions of acetonitrile in the same manner. After each extraction, transfer the clear lower layer to the evaporation flask (Fig. 1 A) and remove the solvent on a flash evaporator. During evaporation the flask is partially immersed in a water bath maintained at 40 ± 2°C.

When all the solvent is removed, dissolve the residue carefully in a few drops of absolute ethyl alcohol. Care should be taken to wash the sides of the flask. Bring final volume to 2 ml in the graduated projection of the flask.

*Thin-layer chromatography.*—Any equipment for the preparation of 8 × 8" TLC plates coated with Silica Gel-G (250 μ) is suitable.

Mark off the glass plates which are coated with Silica Gel-G as shown in Fig. 2. Apply 20, 40, and 60 μl of the sample on separate plates at S and $S_1$. A 50 μl syringe is suitable. Spot a standard mixture of the antioxidants on each of these plates at $K_1$ and develop in

Table 1. Outline of the procedure for colorimetric determinations of antioxidants

| Procedure | 3-BHA | 2-BHA | BHT | PG | NDGA |
|---|---|---|---|---|---|
| 1. Place 5 ml solvent in suction tube | 50% alcohol | 50% alcohol | 100% alcohol | 50% alcohol | 25% alcohol |
| 2. Wash tube with 2 ml solvent | 50% alcohol | 50% alcohol | 25% alcohol | 2.5% $NH_4$ acetate | 25% alcohol |
| 3. Make up volume to 10 ml | 50% alcohol | 50% alcohol | water | 2.5% $NH_4$ acetate | 25% alcohol |
| 4. Add reagents to develop color | 2 ml Borax<br>2 ml CQC | 2 ml BiP<br>2 ml $FeCl_3$ | 2 ml BiP<br>2 ml $FeCl_3$ | 1 ml $FeSO_4$ | 1 ml $FeSO_4$<br>1 ml carb.-bicarb. buffer |
| 5. Reaction time | 15 min. | 30 min. | 30 min.<br>IN DARK | 10 min. | 10 min. |
| 6. Add *n*-butanol | 5 ml | 5 ml | 5 ml | — | — |
| 7. Read absorbance on a suitable spectrophotometer or colorimeter | 620 mμ | 515 mμ | 522 mμ | 515 mμ | 515 mμ |

benzene until the solvent front is about 2 cm from the line CD. After air drying, apply 50 μl sample and standard mixture at S and $K_1$ respectively. Develop the plates in acetonitrile and again air dry. Cover the area XAEC with paper and spray the exposed surfaces, AEDY and CEBZ, with CQC reagent. Locate the antioxidant spots in the area XAEC with reference to the standards.

*Recovery of antioxidants from the TLC plate.*—Place 5 ml appropriate solvent (Table 1) in the tube (Fig. 1 B) and apply suction. By properly positioning the tip end, suck the silicic acid layer into the inner tube. To obtain a consistent area of the silicic acid layer, cut a plastic sheet into rings with an inner diameter of 1". Place the ring on the plate to enclose the area of the spot and pick up all silicic acid inside the area. Immerse the tip in 2 ml of the appropriate solvent to wash the silicic acid onto the fritted disc. Dissolve the antioxidant by applying mild suction alternately on the tip and suction ends. (Five alternate applications of suction were found to be sufficient.) Remove the inner tube and treat the antioxidant solution with appropriate reagents for *Colorimetric analysis.* Rinse the inner tube carefully with up to 1 ml of appropriate solvent.

*Colorimetric analysis.*—The methods were essentially the same as described by Anglin, Mahon, and Chapman (1). A Beckman DU spectrophotometer was used in this study. The analytical procedure is outlined in Table 1.

*Calculations.*—Standard *K* values (absorbance per microgram of the antioxidant): *K* values for each antioxidant were calculated by spotting 20, 40, and 60 μg of the individual antioxidant on a Silica Gel-G plate. The spots were extracted and developed in the manner described earlier. This was considered necessary as some of the reactions involved are influenced by the pH of the medium (6). However, recoveries of the antioxidants from the plate as compared to direct estimation were in the range of 98-100%. The respective concentrations of the antioxidant in the aliquot of fat used are obtained by dividing the observed absorbance by the *K* value.

*Example* (BHA):

Sample (fat): 10 g
Final volume in evaporation flask: 2 ml
Spot application (TLC): 0.05 ml

Absorbance (BHA) 0.108
K BHA 620: 0.0013

$$\% \text{ BHA} = \frac{0.108 \times 2 \times 100}{0.0013 \times 0.05 \times 10 \times 10^6} = 0.01004$$

**Table 2. Recovery of antioxidants**

| Antioxidant Added to Lard, % | | Antioxidant Found, % | Recovery, % |
|---|---|---|---|
| **Individual** | | | |
| BHA[a] | 0.005 | 0.0044 (3-BHA) + 0.0006 (2-BHA) | 100 |
| | 0.010 | 0.0091 (3-BHA) + 0.0010 (2-BHA) | 101 |
| | 0.020 | 0.0180 (3-BHA) + 0.0017 (2-BHA) | 98 |
| 3-BHA | 0.010 | 0.0102 | 102 |
| | 0.020 | 0.0196 | 98 |
| 2-BHA | 0.010 | 0.0098 | 98 |
| | 0.020 | 0.0200 | 100 |
| BHT | 0.005 | 0.0048 | 96 |
| | 0.010 | 0.0101 | 101 |
| | 0.020 | 0.0197 | 98 |
| PG[b] | 0.005 | 0.0051 | 102 |
| | 0.010 | 0.0090 | 90 |
| NDGA[b] | 0.005 | 0.0040 (+0010)[c] | 100 |
| | 0.010 | 0.0082 (+0013)[c] | 95 |
| **Mixtures** | | | |
| 1. PG | 0.010 | 0.0092 (+0.0006)[c] | 98 |
| NDGA | 0.010 | 0.0086 (+0.0011)[c] | 97 |
| 2. 3-BHA | 0.010 | 0.0101 | 101 |
| 2-BHA | 0.010 | 0.0098 | 98 |
| 3. BHA[a] | 0.007 | 0.0063 (3-BHA) + 0.006 (2-BHA) | 98 |
| BHT | 0.007 | 0.0068 | 97 |
| PG[b] | 0.005 | 0.0047 | 94 |
| NDGA[b] | 0.005 | 0.0048 | 96 |
| 4. BHA[a] | 0.010 | 0.0089 (3-BHA) + 0.0011 (2-BHA) | 101 |
| | | 0.0088 (3-BHA) + 0.0010 (2-BHA) | 99 |
| | | 0.0090 (3-BHA) + 0.0010 (2-BHA) | 100 |
| BHT | 0.010 | 0.0098 | 98 |
| | | 0.0097 | 97 |
| | | 0.0099 | 99 |
| PG[b] | 0.007 | 0.0069 | 98 |
| | | 0.0068 | 97 |
| | | 0.0069 | 98 |
| NDGA[b] | 0.007 | 0.0068 | 97 |
| | | 0.0067 | 96 |
| | | 0.0068 | 97 |

[a] Commercial BHA is a mixture of 3-BHA and 2-BHA. Each isomer is determined separately.
[b] PG and NDGA often streak on TLC. These spots and streaks are visible on the plate without spraying and can be effectively picked up.
[c] Figures in parentheses account for the streaks obtained with PG and NDGA.



## Results and Discussion

The four antioxidants were added to 100 g samples of antioxidant-free lard, individually as well as in mixtures. The amounts used, shown in Table 2, were in the range permitted in fats and oils. Results of analysis show recoveries of BHA and BHT in the ranges 98–101% and 96–102%, respectively. PG and NDGA gave streaks, obviously because of the impurities associated with the antioxidants. The spots and streaks of these antioxidants are visible without spraying and the entire area can be picked up effectively to give recoveries up to 98%. Reproducibility of the method was tested and found satisfactory by applying a mixture of the four antioxidants (Mixture 4, Table 2) on three separate plates.

Commercial BHA is a mixture of 3-*tert*-butyl-4-hydroxyanisole (3-BHA) and 2-*tert*-butyl-4-hydroxyanisole (2-BHA) and may contain from 5 to 37% of 2-BHA. 3-BHA is more effective as an antioxidant than 2-BHA (7, 8). Chapman and Mahon reported a method for the estimation of isomers in commercial BHA (9) based on relative absorbances of the mixture obtained with 2,6-dichloroquinonechloroimide-borax reagent and ferric chloride-bipyridine reagent. The proportions of the two were calculated by difference. In the present investigation it was found that the isomers can be quantitatively separated by thin-layer chromatography. BHA used in this study contained 10.5% 2-BHA (Table 2).

The advantages of the above method are that (*1*) all four antioxidants can be estimated in one aliquot of the fat, (*2*) PG and NDGA can be estimated when present together in mixtures, and (*3*) the isomers of BHA can be effectively separated. The data demonstrate that this is a decided improvement over accepted methods of analysis of antioxidants.


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The evaporation flask and the suction assembly were fabricated in the Food and Drug Laboratory by R. G. Campbell.

BHA, BHT, and PG used in the study were obtained from Eastman Chemical Products, Inc.; NDGA was a commercial product of Wm. Stange & Co.


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# Effects of Antioxidants, DPPD and BHT, on Health, Production and Reproduction of Laying Hens[1]

T. E. SHELLENBERGER, D. B. PARRISH AND P. E. SANFORD
*Kansas Agricultural Experiment Station, Manhattan*

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POTTER *et al.* (1956) reported that addition of DPPD (di-phenyl-p-phenylenediamine) to a semi-purified broiler diet low in vitamin A, containing alfalfa meal as the sole source of vitamin A activity, produced substantial increases in vitamin A and carotenoids of the blood plasma and liver of the birds but addition of BHT (dietertiary-butyl-para-cresol) resulted in relatively small increases. Matterson *et al.* (1955) reported that, at the levels of vitamin A studied, storage of the vitamin in the liver was enhanced two to five times when DPPD was in the feed, and plasma vitamin A also was increased but not so markedly. Those workers believed that the increased storage resulted from better utilization of the vitamin after consumption, not simply from protection in the feed. Quisenberry and Matterson (1955), however, found that DPPD in the feed had little effect on pigmentation of broilers. Likewise, Parrish and Shellenberger (1956) found that the inclusion of DPPD in a broiler feed resulted in no consistent or substantial increases in pigmentation or in liver storage of vitamin A.

Oser and Oser (1956) observed that DPPD at dietary levels only several times in excess of those used for the prevention of encephalomalacia in chicks induced prolonged gestation in rats, associated with high mortality of both the overdeveloped pups at birth and the mother during or after parturition.

Since published information on the effect of prolonged antioxidant feeding to laying hens and on hatchability of their eggs was not found, a study of this problem was undertaken and is reported here.

## EXPERIMENTAL

Forty White Leghorn pullets, raised in batteries on wire floors, were randomized into four groups at 18 weeks of age and fed diets containing no antioxidant, 0.125% DPPD, 0.125% BHT, or 0.025% DPPD. All feeds were mixed monthly and contained 2,000 units of vitamin A activity per pound, supplied as carotene in alfalfa meal. The feeds were composed of carotenoid-free ingredients as follows: White corn 60%, soybean oil meal 22%, wheat shorts 8%, non-fat dry milk solids 2%, brewer's yeast 2%, bone meal 2%, $CaCO_3$ 1%, NaCl 0.5%, Wesson oil 0.5%, and vitamin, manganese sulfate and antioxidant premixes in wheat shorts 2%. Feeds were mixed at not longer than monthly intervals to insure freshness. Feeds were adequate in the required micronutrients. The experiment was terminated when the birds reached 1 year of age.

## RESULTS AND DISCUSSION

When the four experimental feeds were analyzed for carotene (Assoc. Offic. Agri. Chemists, 1955) at 8- and 11-month ex-

[1] Contribution No. 560, Department of Chemistry, and No. 233, Department of Poultry Husbandry, Kansas Agricultural Experiment Station, Manhattan.

perimental periods, one month after mixing in each instance, it was found that the feeds including the one not containing an antioxidant, had more than 1,800 units of vitamin A activity, and all had a similar carotenoid pigment content.

Three months after the pullets came into production, eggs were collected and the yolks analyzed for carotenoids and vitamin A (Table 1). The carotenoid levels were higher in eggs from hens receiving antioxidants. Vitamin A levels in the eggs were higher when DPPD was fed but were little affected when BHT was fed. Higher carotenoid but lower vitamin A levels were found in eggs from the 0.025% DPPD group than in those from the 0.125% DPPD group. Similar data were obtained at 4½ months and 7 months of production but the differences among the groups were not so great. However, the vitamin A content of eggs from the BHT group at 4½ months of production was 25% lower than that of the control group.

Liver and blood serum samples were taken at the termination of the experiment and analyzed for carotenoids and vitamin A (Table 2). Samples from hens receiving antioxidants in the feed contained more carotenoids and more vitamin A than those from controls. Since all feeds had similar carotenoid and vitamin A activities when mixed, and also one month after mixing as mentioned previously, the data suggest that these antioxidants increased body and egg stores of carotenoids and vitamin A in some unknown manner after the feed had been consumed.

After 4 months of production, the pullets were artificially inseminated and eggs were collected during a 10-day period to study effects of dietary DPPD and BHT on hatchability; 246 unbroken eggs were collected. Fertility was checked on the 18th day of incubation, and the 233 fertile eggs were transferred for hatching. Fertility was 92% or more in all groups. Hatchability of fertile eggs of Group 1 (control) was 89.5%, Group 2 (0.125% DPPD) 93.5%, Group 3 (0.125% BHT) 87.0%, and Group 4 (0.025% DPPD) 98.5%. Thus, the presence of DPPD and BHT had no apparent harmful effect on hatchability of eggs. Early viability of the chicks was excellent. Only one chick was lost (accidental cause) during the first two weeks after hatching, at which time this study was terminated with all chicks apparently in normal condition. Growth of chicks of all 4 groups was normal.

Production records on a hen-day basis for the 7 months of lay were 64.8%, 67.5%, 62.2%, and 72.5%, respectively, for Groups 1, 2, 3, and 4. Birds were not culled from the experiment until dead or so near death that recovery was impossible. Some birds were out of production three to four weeks before death but were included when calculating production rec-

TABLE 1.—*Average carotenoid and vitamin A content of egg yolks*

| Group | Treatment | Carotenoids[1] | | Vitamin A | | Stage of production[2] |
|---|---|---|---|---|---|---|
| | | mmg./g. | mmg./yolk | mmg./g. | mmg./yolk | |
| 1 | Control | 5.4 | 82.9 | 3.0 | 45.8 | 54.6 |
| 2 | 0.125% DPPD | 7.6 | 111.6 | 4.8 | 72.3 | 60.6 |
| 3 | 0.125% BHT | 7.5 | 108.2 | 3.0 | 42.7 | 55.2 |
| 4 | 0.025% DPPD | 8.0 | 114.1 | 4.1 | 58.2 | 56.8 |

[1] Carotenoids read as carotene.
[2] Average number of eggs laid since start of production. One egg analyzed from each of 5 hens per group.

TABLE 2.—*Average carotenoid and vitamin A in blood serum and liver from hens at termination of experiment*[1]

| Group | Treatment | Blood serum | | Liver | | | |
|---|---|---|---|---|---|---|---|
| | | Carotenoids, mmg./100 ml. | Vitamin A, mmg./100 ml. | Carotenoids, mmg./g. | Carotenoids, mmg./liver | Vitamin A, mmg./g. | Vitamin A, mmg./liver |
| 1 | Control | 27.5 | 18.6 | 1.0 | 34.3 | 0.4 | 12.7 |
| 2 | 0.125% DPPD | 49.7 | 32.0 | 2.6 | 90.0 | 1.7 | 65.1 |
| 3 | 0.125% BHT | 73.8 | 25.1 | 2.5 | 71.9 | 0.9 | 26.5 |
| 4 | 0.025% DPPD | 53.5 | 31.5 | 3.0 | 86.5 | 1.1 | 29.7 |

[1] Two hens per group. Hens had high and similar production records.

ords. Neural and visceral leucosis was responsible for a loss of 25% of birds used on this study.[1] "Cage fatigue" was apparently the cause of most of an additional 25% loss. This syndrome was most prevalent in the control group during the seventh month of production; otherwise losses were similarly distributed among the 4 groups.

Feed conversions (lbs. feed/doz. eggs) were 3.68, 4.31, 4.06, and 3.64 for Groups 1, 2, 3, and 4, respectively. The birds receiving high levels of antioxidant (Groups 2 and 3) had the poorest feed conversions; however, these birds put on more weight throughout the production period than either of the other groups. Adjustment in feed consumption was not made for feed eaten by sick birds. Birds of Group 4 (0.025% DPPD) increased in weight somewhat during the latter part of production. Group 1 hens (control) consistently lost weight, the loss leveling out during the last 4–5 months. Eggs from birds of Group 2, despite heavier production, were slightly larger than those obtained from the other groups.

Eggs laid during the last week of each month of production were examined for blood spots and other abnormalities. In Group 1, 8.2% had blood spots; in Group 2, 7.3%; in Group 3, 13.8%; and in Group 4, 4.4%. Other abnormalities, only occasionally observed, were distributed among all experimental groups.

Eggs from Group 2 (0.125% DPPD) were checked qualitatively for presence of DPPD by a modification of the method of Smith *et al.* (1955). Only a trace was found. Blood serum from birds of this group was tested but no DPPD was detected. A liver sample from the same birds had only a trace of DPPD, if any. As expected, a qualitative check for DPPD in the feces of both DPPD groups gave a strong positive test.

These results suggest that further consideration should be given to the effects of the use of antioxidants in feeds for commercial egg production.

## SUMMARY

A study was made of the effects of the addition of the antioxidants DPPD and BHT to feeds on health, production and reproductive performance of laying hens. Vitamin A and carotenoid pigment contents of eggs, liver, and blood serum also were determined. These antioxidants did not have an adverse effect on the hens. Vitamin A and carotenoid pigment contents were higher in eggs, liver and blood serum of hens getting the antioxidants.

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# SUMMARY OF TOXICOLOGICAL DATA

## DISPOSITION OF BUTYLATED HYDROXYTOLUENE (BHT) IN THE RAT

R. TYE, J. D. ENGEL and I. RAPIEN

*The Kettering Laboratory, University of Cincinnati, Cincinnati, Ohio, USA*

*(Summary* of unpublished report dated 2 October 1962)*

### *Introduction*

The present investigation was undertaken with the primary objective of comparing the characteristics of the absorption, distribution, storage and excretion of butylated hydroxytoluene (BHT; 3,5-di-*tert*-butyl-4-hydroxytoluene) by rats, with those of a related compound. The $^{14}C$ isotope of BHT labelled in the $\beta$-position of the *tert*-butyl groups was used throughout the present experiments.

### *Absorption and excretion*

*Oral route.* Pairs of young, adult, albino rats of the Nelson strain (1 male and 1 female per pair) were given 1–5 oral doses of about 0·2 mmoles [$^{14}C$]BHT/kg, i.e. about 44 mg/kg, on alternate days commencing day 0 and ending on day 9. Daily elimination of $^{14}C$ in the urine and faeces was followed until the pairs of rats were killed off on days 1, 3, 6 and 8, i.e. 24 hr after the administration of the respective terminal doses. However, after the fifth and final dose, the last remaining pair of rats was kept alive for a further period of 8 days (until day 17), during which the daily excretion of $^{14}C$ was also recorded.

Table 1. *Fate of repeated oral doses of [$^{14}C$]BHT (0·2 mmoles/kg) in the rat*

| No. of doses | Percentage of total dose in | | | | | | | | Percentage of total dose accounted for | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Faeces | | Urine | | Gastro-intestinal tract | | Body | | | |
| | M | F | M | F | M | F | M | F | M | F |
| 1 | 57·8 | 22·6 | 2·8 | 18·6 | 27·5 | 35·5 | 4·4 | 15·9 | 92·0 | 92·6 |
| 2 | 69·6 | 30·9 | 6·2 | 36·5 | 23·2 | 17·5 | 4·5 | 8·8 | 103·5 | 93·7 |
| 3 | 79·5 | 45·9 | 4·0 | 25·4 | 12·9 | 18·5 | 2·3 | 6·8 | 98·7 | 98·6 |
| 4 | 82·5 | NR | 3·9 | NR | 10·0 | NR | 2·5 | NR | 98·9 | NR |
| 5 | 76·9 | 50·6 | 15·1 | 43·1 | 0·4 | 3·7 | 0·3 | 0·6 | 92·7 | 98·0 |

M = Male F = Female NR = No result due to escape of animal

From Table 1 it can be seen that substantial amounts of $^{14}C$ were eliminated from the body via the urine and faeces. A negligible amount (0·05%) of the dose was exhaled from the lungs of 2 males in 24 hr following the administration of a dose of 0·2 mmoles/kg. Moderate amounts of $^{14}C$ were still present in the gastro-intestinal tract, but comparatively

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Table 2. *Temporal course of elimination of $^{14}C$ in faeces and urine of rats given 1–5 oral doses of [$^{14}C$]BHT (0·2 mmoles/kg)*

| No. of doses | Percentage of dose excreted on days | | | | | | | | | | | | | | | | Average† percentage of total dose excreted |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1–2 | | 3–5 | | 6–7 | | 8–9 | | 10–12 | | 13–14 | | 15–16 | | 17 | | |
| | F | U | F | U | F | U | F | U | F | U | F | U | F | U | F | U | |
| **Males** | | | | | | | | | | | | | | | | | |
| 1 | 84·8* (27·5) | 2·8 | | | | | | | | | | | | | | | 88 |
| 2 | 67·1 | 7·4 | 93·1* (46·3) | 4·9 | | | | | | | | | | | | | 86 |
| 3 | 63·0 | 4·3 | 92·0 | 5·9 | 122·4* (38·8) | 1·8 | | | | | | | | | | | 63 |
| 4 | 72·6 | 3·9 | 86·3 | 6·1 | 93·8 | 3·7 | 117·6* (40·1) | 1·8 | | | | | | | | | 97 |
| 5 | 66·8 | 8·1 | 75·9 | 18·7 | 67·9 | 9·7 | 76·3 | 12·3 | 67·8 | 15·6 | 24·6 | 6·7 | 3·4 | 2·4 | 3·2* (1·8) | 1·9 | 92 |
| Average† | 71 | 5 | 87 | 9 | 95 | 5 | 97 | 7 | | | | | | | | | 85 |
| **Females** | | | | | | | | | | | | | | | | | |
| 1 | 58·1* (35·5) | 18·6 | | | | | | | | | | | | | | | 77 |
| 2 | 33·6 | 34·4 | 63·2* (35·0) | 38·6 | | | | | | | | | | | | | 85 |
| 3 | 32·6 | 24·9 | 63·6 | 35·4 | 96·8* (55·5) | 16·1 | | | | | | | | | | | 90 |
| 4 | NR | 13·9 | NR | NR | NR | NR | NR | NR | NR | NR | NR | NR | NR | NR | NR | NR | NR |
| 5 | 41·1 | 26·4 | 47·1 | 54·9 | 36·6 | 31·0 | 53·3 | 31·3 | 52·5 | 50·1 | 16·5 | 14·0 | 2·5 | 5·3 | 22·0* (18·6) | 2·4 | 97 |
| Average† | 43 | 24 | 58 | 43 | 67 | 24 | | | | | | | | | | | 87 |

F=Faeces U=Urine NR=No result due to escape of animal.
*Total value, including $^{14}C$ content of gastro-intestinal tract 24 hr after dosing; this amount is shown in brackets.
†Average values are expressed to nearest whole number.

small amounts were retained elsewhere in the body. Quantitative recoveries of the total $^{14}C$ administered were obtained. It is noteworthy that the fate of BHT in the body was the same, irrespective of the number of doses administered.

Table 1 also reveals a striking sex difference in the main pathways for the elimination of BHT. Whereas, females excreted considerable quantities in their urine (19–43% of the dose), males put out as much as 58–83% of the dose in the faeces but only 3–15% in the urine. Despite the greater absorption of BHT by females, both sexes readily eliminated the antioxidant from the body.

Table 3. *Excretion of $^{14}C$ in urine and faeces of rats given single subcutaneous injections of [$^{14}C$]BHT*

| Dose (mmoles/kg) | Percentage of dose excreted in | | | | Percentage of total dose excreted |
|---|---|---|---|---|---|
| | Faeces | | Urine | | |
| | 0–2 days | 2–4 days | 0–2 days | 2–4 days | |
| 0·0003 | 40 | 37 | 11 | 3 | 91 |
| 0·0022 | 26 | 12 | 7 | 3 | 48 |
| 0·025 | 24 | 6 | 4 | 2 | 37 |
| 0·22 | 20 | 9 | 5 | 2 | 36 |

Details of the daily faecal and urinary output of BHT are shown in Table 2. As already indicated in Table 1, there was no tendency on the part of either sex for the total output of $^{14}C$ to drop with increasing number of doses. After the fifth and concluding dose, the expected decline in the amount of excreted $^{14}C$ ensued. By day 17, up to 92% by males, and 97% by females, of the total BHT administered, had been eliminated from the body.

*Subcutaneous route.* In view of the high faecal output of the antioxidant, the subcutaneous route was employed to seek the main excretory pathway of that part of the dose that had been absorbed into the body. The study was limited to 4 female rats of the Charles River CD strain to which were given single, subcutaneous injections of 0·0003, 0·0022, 0·025 and 0·22 mmoles/kg in peanut oil. Urinary levels of $^{14}C$ were estimated 2 and 4 days after the administration of the material. In direct contrast to the oral route, females excreted the majority of the dose (29–77%) in the faeces and small amounts (6–14%) in the urine (Table 3). Increasing the dose resulted in a lowering of the relative rate of excretion of $^{14}C$, an effect which was reflected equally in the faeces and urine.

### *Distribution and storage*

Employing the same schedule of oral dosage as described above, the distribution and storage of [$^{14}C$]BHT were determined in the tissues of the rat (Table 4). The highest concentrations of $^{14}C$ were detected in liver, kidneys, lungs and body fat. Notably low levels were present in the brain, blood and testes. In general, females retained more $^{14}C$ in the tissues than males, a difference which was especially marked in the gonads. Concentrations of $^{14}C$ in tissues were not related to the number of doses administered, and hence there was no accumulation of $^{14}C$ in the tissues on repeated administration. In fact a similar pattern of the distribution of $^{14}C$ in the tissues emerged the day after each dose was given. With the exception of the skin and body fat, the majority of the tissues of rats, killed 8 days after the fifth and final dose, contained less $^{14}C$ than the tissues of rats killed only 24 hr after this dose had

Table 4. *Percentage distribution of $^{14}C$ in tissues following repeated oral doses of [$^{14}C$]BIIT (0·2 mmoles/kg)*

| No. of doses | Percentage of single dose per g tissue | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Liver | Kidneys | Brain | Lungs | Heart | Gonads | Adrenals | Spleen | Blood | Skin | Muscle | Fat |
| | | | | | | Males | | | | | | |
| 1 | 0·050 | 0·012 | 0·0017 | 0·013 | 0·006 | 0·004 | 0·021 | 0·005 | 0·010 | 0·017 | 0·003 | 0·023 |
| 2 | 0·100 | 0·025 | 0·0034 | 0·011 | 0·016 | 0·007 | 0·016 | 0·009 | 0·015 | 0·025 | 0·009 | 0·069 |
| 3 | 0·086 | 0·014 | 0·0017 | 0·015 | 0·010 | 0·005 | 0·033 | 0·008 | 0·015 | 0·019 | 0·004 | 0·044 |
| 4 | 0·088 | 0·023 | 0·0057 | 0·023 | 0·013 | 0·006 | 0·022 | 0·009 | 0·017 | 0·042 | 0·005 | 0·065 |
| 5* | 0·008 | 0·003 | 0·0004 | 0·004 | 0·003 | 0·0003 | 0·012 | 0·006 | 0·012 | 0·004 | 0·001 | 0·009 |
| | | | | | | Females† | | | | | | |
| 1 | 0·137 | 0·039 | 0·0095 | 0·059 | 0·039 | 0·056 | 0·065 | 0·015 | 0·051 | 0·120 | 0·020 | 0·161 |
| 2 | 0·162 | 0·042 | 0·0091 | 0·045 | 0·040 | 0·150 | 0·089 | 0·016 | 0·066 | 0·117 | 0·033 | 0·123 |
| 3 | 0·135 | 0·038 | 0·0095 | 0·067 | 0·033 | 0·077 | 0·196 | 0·016 | 0·052 | 0·185 | 0·019 | 0·243 |
| 5* | 0·011 | 0·007 | 0·0010 | 0·013 | 0·005 | 0·016 | 0·012 | 0·010 | 0·021 | 0·018 | 0·002 | 0·054 |

*Animals were killed off 8 days after fifth dose, other animals were killed 24 hr after receiving their final dose.
†No results for females on 4 doses because of escape.

been administered. This observation may be attributed to the continued excretion of gradually declining amounts of $^{14}C$ in the 8-day period after the fifth dose, as shown in Table 2.

*Summary and conclusion*

Repeated oral doses of [$^{14}C$]BHT did not influence the rapid rate of elimination of $^{14}C$ from the bodies of male and female rats. The main excretory pathway was that of the faeces but the urinary route was far more important in females than in males. The greater absorption of [$^{14}C$]BHT by females was reflected in the higher levels of $^{14}C$ in their tissues, as compared with males. There was no evidence of accumulation of [$^{14}C$]BHT in the body under the conditions of repeated dosage employed in these experiments.

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tumors. This report on the first four compounds is part of a study including eighteen related simple aromatic amine derivatives. It is concluded that even monocyclic aromatic amines may be carcinogenic.

15. *Antioxidants and Carcinogenesis: Butylated Hydroxytoluene, but not Diphenyl-p-phenylenediamine, Inhibits Cancer Induction by N-2-Fluorenylacetamide and by N-Hydroxy-N-2-Fluorenylacetamide in Rats.* B. M. ULLAND, J. H. WEISBURGER, R. S. YAMAMOTO, and E. K. WEISBURGER, Bionetics Research Laboratories, Inc., Kensington, Maryland, National Cancer Institute, Bethesda, Maryland.

In continuation of our studies on inhibition of cancer induction with the carcinogens *N*-2-fluorenylacetamide (FAA) and the *N*-hydroxy derivative (NOHFAA) by acetanilide and related compounds, we now report on the effect of the antioxidants butylated hydroxytoluene (BHT) and diphenyl-*p*-phenylenediamine (DPPD). Groups of rats were fed 223 ppm FAA or 239 ppm NOHFAA (1 mmol) carcinogen and a 30 mole excess, namely 7850 ppm DPPD or 6600 ppm BHT, in Wayne Chow for 24 wk (male Charles River strain rats) or 32 wk (female rats). Male and female rats continued on control diet for another 12 wk. With FAA alone 70% of males had hepatoma, 20% of females mammary adenocarcinomas. With NOHFAA, 60% of male rats had hepatoma, and 70% of females had mammary adenocarcinoma. The simultaneous administration of DPPD failed to alter induction of cancer in male or female rats. However, BHT reduced the incidence of hepatoma in males to 20% when the carcinogen was FAA. With NOHFAA, BHT lowered the liver tumor incidence to 15% and the mammary cancer incidence in females to 35%. Similar data on the inhibition by BHT were obtained utilizing different levels of carcinogens in Fischer strain rats. Equimolar levels of sulfate failed to increase liver tumor incidence in animals given NOHFAA and BHT. Also, BHT failed to depress the excretion of free urinary inorganic sulfate. Liver and esophageal tumor induction with 51 ppm diethylnitrosamine in drinking water for 24 wk was not affected by BHT or DPPD. Thus, BHT, but not DPPD, inhibits cancer induction by FAA and NOHFAA by as yet unknown mechanisms.

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## SHORT PAPERS

# The Transfer of Dietary Butylated Hydroxytoluene (BHT) into the Body and Egg Fat of Laying Hens

P. G. C. VAN STRATUM and H. J. VOS

*Central Institute for Nutrition and Food Research T.N.O., Zeist, Netherlands*

(*Received* 27 *May* 1965)

Abstract—Laying hens fed 500 ppm of butylated hydroxytoluene (BHT; 3,5-di-*tert*-butyl-4-hydroxytoluene) in the diet, produced eggs with 20 ppm of the antioxidant in the fat fraction. Fifty-five ppm of BHT was found after 21 wk in the body fat. A dietary level of 100 ppm BHT resulted in less than 5 ppm in the egg fat and in body fat values of 5 ppm or less. At lower dietary concentrations of BHT neither the egg fat nor the body fat tissues contained detectable amounts of unchanged BHT. It is concluded that normal levels of BHT in feedstuffs will not cause retention of unchanged antioxidant in egg or body tissues of laying hens.

Since butylated hydroxytoluene (BHT; 3,5-di-*tert*-butyl-4-hydroxytoluene), which is widely used as an antioxidant for the stabilization of lipids in animal feedstuffs, accumulates in the tissue fat of chickens fed on BHT in the diet (Francois & Pihet, 1960), the possible retention of unchanged antioxidant in the body fat and eggs of laying hens that had been treated orally has now been investigated.

Individually housed laying birds were fed on a standard diet with the following percentage composition: maize meal, 32·8; ground wheat, 8·0; ground oats, 10·0; soya-bean meal, 20·0; herring meal, 6·0; meat scraps, 3·0; lucerne meal, 4·0; dried whey, 3·0; mineral mixture, 4·0; degummed soya-bean oil, 5·0; vitamin additives, 4·2. The hens were allowed free access to water and ground oyster shell. After adaptation, 3 groups of 4 birds were fed on the standard diet which was supplemented respectively with 0 (controls), 5 and 50 ppm of BHT, the antioxidant being dissolved in the soya-bean oil component of the ration. After 8 wk, the dietary concentrations of BHT were changed respectively to 0 (controls), 100 and 500 ppm.

The birds remained in good health throughout the 29 wk on test. Egg weights and egg production were not significantly influenced by treatment with BHT, and this finding is in agreement with the work of Shellenberger, Parrish & Sanford (1957) who showed that the health, production and reproductive performance of laying hens were not affected by the daily intake of 1250 ppm of BHT in the diet during 8 months.

At 0, 4, 8, 10, 12, 16 and 20 wk, one egg was collected from each hen, and the mixed yolks from the 4 eggs of each group were analyzed for BHT. The residual lipids from the boiling ethanol–chloroform extracts were fractionated by column chromatography on silicic acid, using isooctane as the eluent, and ultraviolet absorption spectral measurements were made of the eluate between 270 and 340 mμ using an appropriate blank prepared from the egg yolks of untreated hens. The BHT content was estimated by the method of Vos (1959). At 26 wk, BHT was determined in the lipids of 4 eggs from each hen. The results are summarized in Table 1.

Table 1. *BHT content of egg fat of laying hens fed various levels of BHT*

| vel of BHT pm) | BHT in egg fat (ppm) at wk | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 | 4 | 8 | 10 | 12 | 16 | 20 | 26 |
| 8 wk then a thereafter | 0 | 0 | 0 | 0 | 0 | 0 | 0 | T |
| r 8 wk then a thereafter | 0 | 0 | 0 | 24 | 20 | 18 | 18 | 24* |

*SE=±3.

separate hens at 26 wk in ppm: 100-ppm group: trace <5, 0, 0, trace; 500-ppm group: 33, 21,

T was found in the egg fat of hens with a dietary intake of 5 or 50 ppm BHT over ding at 500 ppm resulted in the accumulation of 20 ppm of the antioxidant in the after 2 wk, and this concentration remained roughly constant during the following e lowest dietary concentration of BHT, which caused detectable amounts of the it to become apparent in the egg fat, was 100 ppm. The lipid component of this % of soya-bean oil) contained 2000 ppm of BHT, i.e. 20 times the concentration ommended for stabilizing fats and oils. If lipids, stabilized with normal concen- BHT, are incorporated into the rations of laying hens, it is unlikely that detect- nts of unchanged antioxidant would be found in the fat of the eggs.

wk, the birds were killed, and the solvent extracts of the fatty tissues were r the antioxidant; the fats of the control hens being used for the preparation of he group fed on 500 ppm BHT, 2 samples of body fat contained 56 and 52 ppm of unchanged antioxidant; these concentrations are approximately one-half of nended for the stabilization of food fats. These results agree with the value of 60 T which was found in the body fat of broiler chickens, fed 1000 ppm BHT for 8 is & Pihet, 1960). In the group of laying hens fed 100 ppm BHT, 2 samples of tained <5 and 5 ppm of BHT; these values approach the lowest concentrations y our analytical methods. Again, the concentration of BHT in the lipid compo- mixed diet was 20 times that recommended for practical usage, and it might be normal stabilization with BHT of the lipid components of feedstuffs would not on of unchanged antioxidant in the tissue of laying hens.

*nts*—The sample of BHT ("Ionol") was kindly supplied by Shell Nederland Chemie N.V. at

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## Passage du butylate d'hydroxytoluène alimentaire (BHT) dans le corps et le jaune des oeufs de poules pondeuses

**Résumé**—Des poules pondeuses auxquelles on a fait absorber 500 ppm de butylate d'hydroxytoluène (BHT; 3,5-di-*tert*-butyl-4-hydroxytoluène) mélangés aux aliments, ont pondu des oeufs dont les jaunes contenaient 20 ppm du produit antioxydant. On a retrouvé 55 ppm de BHT dans les graisses du corps de la poule au bout de 21 semaines. A la dose de 100 ppm de BHT, on a retrouvé moins de 5 ppm dans le jaune d'oeuf et, dans les graisses du corps, 5 ppm ou moins. Pour des concentrations alimentaires inférieures de BHT, ni le jaune d'oeuf ni les tissus adipeux de la poule ne contenaient de quantités décelables de BHT non transformé. On en conclut que des quantités normales de BHT dans la nourriture ne provoqueront pas de rétention d'antioxydant non transformé dans l'oeuf ou dans les tissus des poules pondeuses.

## Der Übergang von verfüttertem butyliertem Hydroxytoluol (BHT) in Körper und Eifett von Legehennen

**Zusammenfassung**—Legehennen, die mit dem Futter 500 ppm butyliertes Hydroxytoluol (BHT; 3,5-Di-*tert*-butyl-4-hydroxytoluol) erhielten, legten Eier mit 20 ppm des Antioxydationsmittels in der Fettfraktion. Nach 21 Wochen wurden 55 ppm BHT im Körperfett festgestellt. Bei Verfütterung von 100 ppm BHT wurden unter 5 ppm im Eifett und 5 ppm oder darunter im Körperfett gefunden. Bei noch geringerem BHT-Anteil im Futter enthielten das Eifett und die Körperfettgewebe keine feststellbaren Mengen unverändertes BHT. Daraus lasst sich schliessen, dass normale Mengen BHT im Futter keine Retention unveränderten Antioxydationsmittels in den Eiern und den Körpergeweben von Legehennen verursachen.

**12** INHIBITION OF CARCINOGENIC AND TOXIC EFFECTS OF POLYCYCLIC HYDROCARBONS BY PHENOLIC ANTIOXIDANTS AND ETHOXYQUIN. Lee W. Wattenberg. Department of Pathology, University of Minnesota, Minneapolis, Minn. 55455.

The objective was to determine if butylated hydroxyanisol (BHA), butylated hydroxytoluene (BHT) and ethoxyquin could alter carcinogenic and toxic effects of polycyclic hydrocarbons. Breast tumor formation from an oral dose of 12 mg. of DMBA to female Sprague Dawley rats was studied. One hour prior to DMBA, 200 mg. of antioxidant in olive oil or vehicle was given by mouth. 18 weeks later results were (rats with breast tumors/no. at risk): vehicle 27/34 (79%); BHA 7/18 (39%); BHT 5/18 (28%); ethoxyquin 2/17 (12%). The effects of antioxidants on adrenal necrosis in rats receiving 30 mg. of DMBA was determined under similar conditions. Complete protection was obtained with the 3 antioxidants whereas 97% of controls showed adrenal necrosis. Female A/HeJ mice fed diets for 2 weeks containing BP 1 mg/Gm with or without BHA or BHT 5 mg/Gm were studied for tumors of the forestomach. 10 weeks later results were (mice with tumors/no. at risk): no antioxidant 12/12; BHA 2/12; and BHT 2/9. Lethal effects for mice of feeding a diet containing DMBA 0.2 mg/Gm for 4 weeks can also be inhibited by BHA or BHT. The results indicate that the antioxidants employed can inhibit carcinogenic and toxic effects of polycyclic hydrocarbons. (USPHS grant CA09599).

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