THE URINARY EXCRETION OF TRITIATED BUTYLATED HYDROXYANISOLE AND BUTYLATED HYDROXYTOLUENE IN THE RAT

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The total radioactivity of rat urine after the administration of tritiated butylated hydroxyanisole and butylated hydroxytoluene has been measured. The results indicate that approximately 90 per cent of the dose of BHA is excreted within four days. With BHT, the total radioactivity excreted in the same period is equivalent to only 35 per cent of the dose, but some of the radioactivity of the labelled molecule is lost during metabolism and only an approximate estimation of the total urinary excretion is possible.

THE substances butylated hydroxyanisole (BHA) and butylated hydroxy toluene (BHT) are permitted antioxidants in certain foods in many countries so that their method of elimination from animals is a matter of interest. Dacre, Denz and Kennedy (1956) studied the chemical nature of the urinary excretory products of BHA as well as the rate of their excretion in rabbits. About 60 per cent of a single dose of 1 g. was excreted in the urine as glucuronide, ethereal sulphate and free phenol in 24 hr. The dose per cent recovered in the urine in a given time increased with diminishing doses. Dacre (1960) also reported that rats excreted 90 per cent of a single dose (0·08 to 0·1 g.) of BHA in urine. Astill, Fassett and Roudabush (1960) investigated the urinary excretion of BHA in rats at lower dose levels ranging from 2 to 100 mg./kg. and were able to account for 80–100 per cent of the dose as urinary metabolites over a period of about 3 to 6 days. The urinary excretory products of BHT in rabbits were also examined after high doses by Dacre (1961) who estimated that 54 per cent of a single dose could be recovered in the urine in the form of various metabolites over a period of 3 to 4 days.

By the use of BHA and BHT labelled with tritum we have now been able to compare the excretion of these substances in rat urine using a dose of 0·5 mg./kg. which approaches more closely the amounts of these antioxidants encountered in human diets than have previous experiments.

EXPERIMENTAL

Materials

Butylated hydroxyanisole is normally a mixture of two isomers, 85 per cent of 2-t-butyl-4-methoxyphenol and 15 per cent of 3-t-butyl-4-methoxyphenol. A sample of the pure 2-t-butyl isomer obtained from Nipa Laboratories Ltd., Cardiff, Wales, was used in the subsequent experiments. A pure sample of butylated hydroxytoluene, 2,6-di-t-butyl-4-methylphenol was obtained from the same source. Both substances were labelled by Dr. J. Garnett, Chemistry School, University of New South Wales, Sydney, by tritum exchange (Wilzbach, 1957). The tritiated

substances were purified by recrystallisation to constant specific activity, and the homogeneity of each checked by paper chromatography before use. R_F values BHA-^{3}H(2-t butyl isomer) 0·44; BHT-^{3}H 0·94. Descending method, using Whatman No. 1 paper, tanks equilibrated at 20–22°. Solvent systems: organic phase of iso-octane, methanol, water (10:8:2). The specific activity of the BHA-^{3}H(2-t-butyl isomer) was 35 μc/mg. and that of the BHT-^{3}H5 μc/mg.

Tritiated toluene (specific activity $1{\cdot}75 \times 10^6$ d.p.m./ml.) was obtained from the Packard Instrument Co. Inc., La Grange, Illinois, U.S.A. and used as internal standard in the counting of the samples.

Animal Experiments

2-t-Butyl-4-methoxyphenol (97 μg. = $5{\cdot}44 \times 10^6$ d.p.m.) or 2,6-di-t-butyl-4-methylphenol (100 μg. = $1{\cdot}29 \times 10^6$ d.p.m.) were injected in 50 per cent aqueous ethanol intraperitoneally into each rat (albino: average weight 200–250 g.). The rats were placed singly (BHA experiment) and in pairs (BHT experiment) in all-glass metabolic containers fitted with stainless steel mesh gauze to separate the urine from the faeces. The rats were fed with rat cubes once every 24 hr. and water was constantly available during the experiments; the urine was collected quantitatively from the containers every 24 hr. for a period of 4 successive days. The urine was filtered, counted adjusted to a convenient volume with water and three aliquots (0·10 ml.) were counted from each urine sample.

Counting Technique

All radioactive estimations were carried out using a Packard Tricarb Liquid Scintillation Spectrometer, Model 314.

The urine samples were pipetted into a scintillation solvent (10·0 ml.) which contained 3·0 g. 2,5-diphenyloxazole and 0·1 g. of 1,4-bis-2-(5-phenyloxazoyl)benzene dissolved in a litre of toluene-ethanol (4:1 v/v). Readings were taken after refrigeration of the samples to −13° over an average time interval of 10 min. per vial with discriminator settings at 10–50 V, and 10–100 V.

Two assay samples of the injection solution were also taken, the volumes made up to 25·0 ml. with 50 per cent aqueous ethanol and subsequently three smaller aliquots (0·10 ml.) from the diluted solution were counted in the scintillation solvent described above (10·0 ml.).

Quenching due to the presence of urinary pigment and ethanol was compensated for by the addition of a fixed volume of the internal toluene-^{3}H standard (10 μl.) to urine vials, assay sample vials and blank vials (containing only scintillation solvent minus ethanol) and recounting all vials at the same high voltage and discriminator settings for the same time intervals.

Distribution of Tritium in 2,6-di-t-butyl-4-methylphenol (BHT-^{3}*H*)

A sample of the BHT-^{3}H used in the excretory experiments (47·3 mg.) was mixed with 2·0 g. of BHT and steam distilled to give a product which, when recrystallised from benzene-light petroleum to constant count, had

an activity of $6{\cdot}0 \times 10^7$ d.p.m./mM (m.p. 70°). This product (2·0 g.) was oxidised with bromine in 80 per cent v/v acetic acid (Coppinger and Campbell, 1953) to yield 3,5-di-t-butyl-4-hydroxybenzaldehyde-^{3}H (0·8 g., m.p. 189°) which after recrystallisation from methanol to constant count had an activity of $4{\cdot}1 \times 10^7$ d.p.m./mM.

Kuhn-Roth oxidation of this radioactive aldehyde (365 mg.) gave 146 mg. of acetic acid after distillation (estimated by titration with 0·1 N NaOH) which was converted to its *p*-bromophenacyl ester. The ester was recrystallised to constant count from light petroleum to give a product (216 mg.) with an activity of $0{\cdot}087 \times 10^7$ d.p.m./mM (m.p. 86.°)

Results and Discussion

A comparison of the urinary excretion of BHA and BHT based on the total radioactivity of the recovered urine is shown in Table I. With BHA the excretory levels obtained with the tritiated material are similar

TABLE I

URINARY EXCRETION OF BHA-^{3}H* (2-T-BUTYL ISOMER) AND OF BHT-^{3}H† BY RATS

Day	No. of expts. carried out	Mean per cent of radio activity excreted		Limits of error (P = 0·95)	
		BHA-^{3}H	BHT-^{3}H	BHA-^{3}H	BHT-^{3}H
1	6	86·0	12·0	77·0 –94·0	6·0–18·0
2	6	3·0	11·0	1·0 – 5·0	7·0–15·0
3	6	0·6	7·0	0·3 – 0·9	4·0–10·0
4	6	1·5	4·5	0·35– 2·5	3·0– 6·0
Total excretion		91·1	34·5	79·0–103·0	20·0–49·0

* Dose: 97 μg. ($5{\cdot}4 \times 10^6$ d.p.m.).
† Dose: 100 μg. ($1{\cdot}3 \times 10^6$ d.p.m.).

to those found by Astill and others (1960) using unlabelled material at dose levels extending from four to two hundred times as great, and indicate that BHA is most probably almost completely excreted in a short time at very low dose levels. These results support evidence for the safety of BHA as an antoxidant in foods.

Because of the difficulty of chemical estimation the excretion of BHT at very low dose levels has not been previously measured. Our results would indicate that the urinary excretion of BHT is less than that of BHA in rats. This is no doubt due to the lower polarity of BHT and the fact that the phenolic group is not available for conjugation with sulphate or glucuronic acid as it is in BHA (Dacre and others, 1956). However, it is possible to give only an approximate estimate of the urinary excretion of BHT from our results as it is not correct to assume that the total amount of tritium present in the urine after BHT-^{3}H administration is equivalent to the amount of BHT excreted.

Whereas BHA does not undergo metabolic oxidation before conjugation so that there will be no loss of labelled tritium, Dacre (1961) has shown that with BHT, oxidation of the 4-methyl group to the corresponding carboxylic acid and ω oxidation of one of the tertiary butyl groups to a primary alcohol occurs before conjugation. The tritiated BHT used in our

experiments has been shown to have approximately 48 per cent of the total radioactivity associated with the 4-methyl group so that oxidation of this group to carboxylic acid will result in the loss of this amount of radioactivity. The loss of one tritium atom of the tertiary butyl group due to ω oxidation will, however, produce a negligible loss of radioactivity as only 8·7 per cent of the total activity is shared by both tertiary butyl side chains. Thus, if the BHT administered is all oxidised to carboxylic acid during metabolism in the rat, the recovered radioactivity in the urine (Table I) would be equivalent to approximately half the total BHT excreted as metabolites. If, however, the main metabolic route is by ω oxidation of a tertiary butyl group, the radioactivity of the urine would approximate closely to the amount of BHT excreted. According to Dacre (1961) about one third of the urinary metabolites of BHT in the rabbit after high doses is ester glucuronide. If this should hold for the rat at low dose levels, the total radioactivity of the urine would be equivalent to approximately 50 per cent of the administered dose of BHT. This could be verified if ^{14}C-BHT were available and work is proceeding in this laboratory with this end in view.

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