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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 100 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	30·7	4·6	16	<0·01	3·26	40	<0·01
10	0·25	45·4	5·4	36	<0·001	3·93	69	<0·01

* 10 g/day/rat of powdered diet 41B (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

* Diet 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 µmole/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	2·80	146	<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 µmole/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	2·06	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.	2·06	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 fluorimetrically.

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 & Hewgill, 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-Methoxy-biphenyl 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 P<0·001
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.

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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