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of the activity was associated with the soluble fraction.

3. The activity of the thyroid gland, however, was restricted to the soluble fraction.

4. More detailed fractionation studies of cerebral cortex and kidney indicated that the particulate matter associated with enzymic activity corresponded in general to the mitochondrial fraction.

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The Metabolism of Phenolic Antioxidants

2. THE METABOLISM OF BUTYLATED HYDROXYANISOLE IN THE RAT*

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Commercial 'butylated hydroxyanisole' consists of a mixture of 15% or less of 2-*tert.*-butyl-4-hydroxyanisole (isomer A) and 85% or more of 3-*tert.*-butyl-4-hydroxyanisole (isomer B). The introduction of butylated hydroxyanisole as a fat-stabilizer about 10 years ago, and its subsequent extensive use in edible fats, has been accompanied by studies of its acute and chronic toxicity (e.g. Graham, Teed & Grice, 1954). Wilder & Kraybill (1948), found none of the substance, other than questionable trace amounts in depot and carcass of rats receiving 3% of butylated hydroxyanisole in the diet over a prolonged period. Studies on the storage of the compound in dogs given daily doses of 0·3, 30 and 100 mg./kg. for more than a year (Hodge & Fassett, unpublished work) also failed to reveal any butylated hydroxyanisole in perirenal, mesenteric or subcutaneous fat or in the brain, liver and kidney.

The metabolism of butylated hydroxyanisole has been studied in the rabbit by Dacre, Denz & Kennedy (1956), who found that an oral 0·5 g. dose was excreted as glucuronide (60%), ethereal sulphate (12%) and free phenols (4% of the dose). This dosage is very large compared with human intake. The ingestion of phenolic antioxidants as a result of their use in fat stabilization is probably less than 0·1 mg./kg. It is known that variations in dose may alter the proportions of metabolites excreted, where more than one metabolic pathway is available (cf. phenol; Bray, Thorpe & White, 1952). To justify extrapolation from experimental doses to the usage dose, it is desirable to study the pattern of metabolism with a variety of doses, including the lowest possible so that any unsuspected changes in metabolic pattern may be detected. This paper deals in this way with the metabolism in the rat of butylated hydroxyanisole and its component isomers.

METHODS AND MATERIALS

Animals, diets and dosage. Male and female albino rats of Sprague–Dawley strain, average wt. of 128 rats 321 g. (224–443 g.), were fed with Purina Chow and kept singly or

* The paper by Astill, Fassett & Roudabush (1959) is to be regarded as Part 1.

in pairs in metabolism cages. Butylated hydroxyanisole, isomer A or isomer B was administered by oral injection in one of the media shown in Table 5. Control values for metabolites were established by analysis of the pooled urine collected for 1 day before dosing. Urines were analysed immediately or kept frozen until analysis. The total faeces in Expts. 14 and 16 were collected and analysed for unabsorbed butylated hydroxyanisole.

Materials. Butylated hydroxyanisole, m.p. 54–58°, b.p. 264–270° at 733 mm. Hg, isomer A, m.p. 62·5–64·0°, and isomer B, m.p. 64·5°, were kindly supplied by the Tennessee Eastman Co., Kingsport, Tenn., U.S.A., to whom we are indebted for the physical data. Chromatographic analysis showed that isomer A contained 3–5% of *p*-methoxyphenol. The following were purchased: 2:6-dichloroquinonechloroimide, 2:2′-dipyridyl, *tert.*-butylquinol, naphtharesorcinol, D-glucuronolactone (Eastman Organic Chemicals, Rochester, N.Y.), β-glucuronidase (ox-liver; Ketodase, Warner–Chilcott, N.Y.), Safranine bluish (3-amino-7-dimethylamino-5-phenylphenazinium chloride, Allied Chemical and Dye Corp., N.Y.).

Paper chromatography. Table 1 records R_F values, colour reactions and solvent systems: strips of Whatman no. 1 paper were used in the descending method. Isomer A (2–3 μg.) and isomer B (1 μg.) were visually detectable with spray reagents A and C. R_F values of metabolites were determined on the purest available specimens. Chromatograms were scanned at 225 or 275 mμ with an attachment inserted in the cell-holder space of a Beckman model DU spectrophotometer (Table 2). Chromatograms were scanned quantitatively after colour development with a Photovolt Electronic Densitometer model 525 (Photovolt Corp., 95 Madison Avenue, N.Y.).

Estimation of butylated hydroxyanisole on chromatograms. From 5 to 40 μg. of each isomer was chromatographed in solvent II for 2 hr. or solvent III for 1 hr. The dried papers were sprayed with (i) reagent A, and absorbing areas corresponding to butylated hydroxyanisole were scanned at 445 mμ, or (ii) reagent C (Gibbs's Reagent; Mahon & Chapman, 1951), dried and scanned at 595 mμ. Elution of spots located by co-chromatography gave low recoveries with reagent C spectrophotometrically. Results, recorded graphically by plotting extinction against distance along the paper and integrating, showed a linear relation between total extinction of a spot and amount chromatographed. Considerable variation from experiment to experiment was found: thus 20 μg. of isomer B, sprayed with reagent C, gave a mean area in thirteen separate experiments of 249 (212–290) mm.², although good replicates were obtained on co-chromatography. A known amount of the isomer to be determined was co-chromatographed in each determination.

Examination of urine

Paper chromatography. Experimental and control urines (10 μl. each) were compared as in Table 1. Only isomers A and B and a metabolite of isomer A, compound H, could be detected in experimental urines. Scanning of chromatograms in ultraviolet light revealed no metabolites because of absorption by substances of natural origin. Elution and estimation of metabolites located by co-chromatography gave low recoveries.

Ethereal sulphates. The total and inorganic sulphate content was estimated either by Folin's gravimetric or by Sperber's turbidimetric method (Bray & Thorpe, 1954).

Free isomers A and B. Urine (5 or 10 ml.) was gently shaken with ether (60 ml.) for 16–24 hr. The separated ether extract was evaporated to dryness at a temperature below 60–70°. A portion of a solution of the residue in ethanol (0·5 ml.) was chromatographed and the free isomer content was estimated chromatographically. Recoveries from control urine (5 ml.) of added isomer A (0·10–0·72 mg.) were 94 ± 4%, and of added isomer B (0·10–0·78 mg.) were 94 ± 3%. The ethereal sulphates of isomers A and B did not interfere with this estimation, as shown by separate experiments in which 0·15 μmole of isomer A and 0·1 μmole of isomer B were recovered from control urine (10 ml.) to which the corresponding ethereal sulphates (34 μmoles) had been added. In view of the known instability of butylated hydroxyanisole in acid solution (Dacre *et al.* 1956) the usual estimation of combined phenols by acid hydrolysis followed by ether extraction was omitted.

For glucuronides. Total glucuronides were estimated by a modified naphtharesorcinol method (Astill *et al.* 1959), and in low-dose studies by the following procedure. The glucuronide gum obtained by precipitation of the insoluble lead salts at pH 9·5 (as in the procedure of Kamil, Smith & Williams, 1951) from 10 ml. of urine was redissolved in water. A portion, giving an estimated maximum hydrolysis concentration of 0·4 mM of required glucuronide, was added to 5 ml. of 0·2 M-sodium acetate buffer, pH 4·6, and 2 ml. of β-glucuronidase (≡ 10^4 Fishman units, Talalay, Fishman & Huggins, 1946). The mixture was incubated for 16–24 hr. at 37·4°, extracted with ether (2 × 50 ml.) and the extract evaporated and analysed chromatographically for the required isomers. Ether extracts of hydrolysates of similarly treated control urines gave unexpectedly high extinctions at 600 mμ with Gibbs's reagent. This was caused by substances, absent from experimental ether extracts, which gave intense colours with reagent C at R_F 0·4–0·6 (solvent II). Recoveries by the enzymic and chromatographic methods of the glucuronide of isomer B (2·3–19·0 mg., 0·14–0·36 mM in the hydrolysis) added to urine (10 ml.) were 67 ± 4% in nine experiments. Recoveries of this glucuronide (5–20 mg., 0·27 mM in the hydrolysis) from urine without a preliminary lead-salt separation were 24–96% and varied inversely as the volume of urine (2·0–0·4 ml.) in the hydrolysis.

The lead salts of the ethereal sulphates of both isomers precipitated with those of the glucuronides at pH 9·5, but interference with the glucuronide estimation was negligible. Non-enzymic hydrolysis of the ethereal sulphate of isomer A (1·1 mg.) was 1·7% and of that of isomer B (0·95 mg.) was 1·3%. β-Glucuronidase was without action on either sulphate.

Examination of faeces

Total faeces were analysed chromatographically by the ferric chloride–2:2′-dipyridyl method: the excess of reducing power over control faeces was expressed as butylated hydroxyanisole (Astill *et al.* 1959).

RESULTS

Excretion of metabolites by control animals. The average daily excretion by undosed rats was 11·9 mg. (4·8–20·4 mg.) of ethereal sulphates as butylated hydroxyanisole [ethereal sulphate/in-

Table 1. *R_F values and colour reactions of isomers A and B and their metabolites*

Whatman no. 1 filter paper was used throughout in the descending technique. Solvents were: I, organic phase of freshly mixed butanol–acetic acid–water (4:1:5, by vol.); II, organic phase of benzene–acetic acid–water (2:2:1, by vol.); III, organic phase of butanol–formic acid–light petroleum, b.p. 60–75° (1:10:10, by vol.). (1) After 10–15 cm. development; (2) after 35–40 cm. development. Spray reagents were: A, aq. 1% (w/v) silver nitrate mixed with 1 part of aq. 3N-NH_3 soln.; B, equal volumes, freshly mixed, of 1% (w/v) sulphanilic acid in 3N-HCl and aq. 5% (w/v) $NaNO_2$; the dried paper was sprayed with aq. 2% (w/v) Na_2CO_3; C, ethanolic 0·05% (w/v) 2:6-dichloroquinonechloroimide; the dried paper was sprayed with aq. 2% (w/v) sodium borate, or, D, with aq. N-NH_3 soln.; E, N-HCl; the dried paper was heated at 70° for 3 min. and treated as in D. – indicates that no colour reaction was given.

Substance	R_F			Colours given by reagents				
	Solvent I	Solvent II	Solvent III	A	B	C	D	E
Isomer A	0·96	0·92	(1) 0·90 (2) 0·82	Blue–brown	Orange	Blue–grey	Dark blue	–
Isomer B	0·96	0·94	(1) 0·90 (2) 0·90	Blue–black	Orange–red	Blue–purple	Purple	–
2:5-Di-*tert.*-butyl-4-hydroxyanisole	0·95	0·94	(1) 0·94	Weak brown	Weak orange	Pink	–	–
p-Methoxyphenol	0·95	0·80	(1) 0·32	Brown	Red	Blue		
Ethereal sulphate of isomer A	0·82	0·10	(1) 0·00	–	–	–	–	Blue–grey, fades to grey
Ethereal sulphate of isomer B	0·79	0·07	(1) 0·00	–	–	–	–	Bright blue, fades to pink
Glucuronide of isomer A	0·90	0·00	(1) 0·00	–	–	–	–	Very weak blue
Glucuronide of isomer B	0·89	0·00	(1) 0·00	–	–	–	–	Very weak purple
Compound H	0·83	0·00	(1) 0·00	–	Orange	Weak purple	Mauve	Yellow
tert.-Butylquinol	0·97		(1) 0·50	Black	Purple	Blue	Blue–purple	

organic sulphate ratio 0·137 (0·068–0·201)], determined gravimetrically on fourteen groups each of eight rats, and, as determined by the procedures for low doses, on eight groups each of eight rats, 0·15 mg. (0–0·32 mg.) of glucuronides and 0·05 mg. (0–0·11 mg.) of free phenols, as isomer B.

Tables 3–5 record the excretion of metabolites in dose studies.

Metabolism of isomer A. Administration of a single dose of 0·4 g. of isomer A/kg. caused an increase in urinary ethereal sulphate output sufficient to account for 72% of the dose, and a much smaller increase in glucuronide output. About 2% of the dose was recovered from the urine unchanged. Chromatography revealed a minor metabolite, compound H. Repeated doses of 0·5 g./kg. depressed ethereal sulphate output and considerably elevated glucuronide output, the over-all conjugate recovery being slightly lowered. Faecal excretion in repeated-dose studies was 0·2% of the total dose. In Expt. 15, 0·6% of the dose was excreted as the impurity *p*-methoxyphenol and 2·6% as its glucuronide, estimated enzymically and chromatographically.

Metabolism of isomer B. After a dose of 0·4 g./kg., increased glucuronide excretion accounted for 57–71% and increased ethereal sulphate output for 15% of the dose; 4% was excreted unchanged. No minor metabolites were detected. Repeated doses of 0·5 g./kg. lowered the proportion of the dose excreted conjugated. Faecal excretion was 0·3% of the dose.

Metabolism of butylated hydroxyanisole. A single dose of 0·4 g./kg. was metabolized in a similar manner to a single dose of 0·4 g. of isomer B/kg.; no increase in ethereal sulphate output was caused by the 15% of isomer A in butylated hydroxyanisole. Repeated doses of 0·5 g./kg. depressed the proportion of the dose excreted conjugated. With single doses of 2–100 mg./kg., consistently high recoveries of the dose from the urine were found. Excretion of butylated hydroxyanisole was at a maximum on the first day after dosing, and the time for complete excretion of the dose decreased as the dose decreased. The glucuronide conjugation remained the principal metabolic pathway at these doses, accounting for 76% (69–92%) of the dose. The percentage excretion of unchanged butylated

Table 2. *Absorption spectra of some derivatives of isomers A and B*

Solvents were (*a*) ethanol and (*b*) water.

Compound	$\lambda_{max.}$	log ε	$\lambda_{max.}$	log ε
Isomer A (*a*)	225	3·79	288	3·56
Potassium 3-*tert.*-butyl-4-methoxyphenylsulphate (*b*)	225	3·85	276	3·36
Methyl(triacetyl-3-*tert.*-butyl-4-methoxyphenyl-β-glucosid)-uronate (*a*)	225	3·94	280	3·34
3-*tert.*-Butyl-4-methoxyphenyl-β-glucosiduronic acid (*b*)	223	3·89	280	3·36
Isomer B (*a*)	226	3·90	289	3·56
Potassium 2-*tert.*-butyl-4-methoxyphenylsulphate (*b*)	224	3·85	278	3·33
2-*tert.*-Butyl-4-methoxyphenyl-β-glucosiduronic acid (*b*)	224	3·92	280	3·39
Compound H (*b*)	225	—	275	—

Table 3. *Urinary excretion of butylated hydroxyanisole, isomer A and isomer B and their metabolites by rats for 5 days after a single dose of 0·4 g./kg.*

Results are expressed as % of dose fed. Compounds were injected by gastric tube as (*a*) a 50% (w/v) solution in propylene glycol or (*b*) a 10% (w/v) suspension in pea soup (Strained Baby Foods, Beech-Nut Co., Syracuse, N.Y.) diluted with an equal volume of water into each rat in a group of four (Expts. 1–6) or eight (Expt. 7) and urine was collected daily. Values were obtained daily on the pooled daily output of each group. Unless specified otherwise sulphates were measured gravimetrically (Bray & Thorpe, 1954) and glucuronides by the modified naphtharesorcinol method (Astill, Fassett & Roudabush, 1959).

Compound	Expt.	Medium	Average dose (g./rat)	Free butylated hydroxyanisole	Glucuronide	Ethereal sulphate	Total accounted fo
Isomer A	3	*a*	0·09	2	11	73*	86
	4	*b*	0·12	1	4†	61	66
	5	*b*	0·13	2	10†	87	99
Isomer B	2	*b*	0·11	3	71	16	90
	6	*a*	0·11	4	57	15*	76
Butylated hydroxyanisole	1	*a*	0·08	3	61	16*	80
	7	*a*	0·10	7	82	11	100

* Estimated by Sperber's method (Bray & Thorpe, 1954). † Estimated enzymically.

hydroxyanisole in the urine decreased as the dose increased.

Metabolites of isomers A and B

The glucuronide of isomer A could not be isolated, but its crude methyl-triacetyl derivative, after separation on acid-washed alumina, was converted into 3-*tert.*-butyl-4-methoxyphenyl-β-glucuronamide, identical with a synthetic specimen. The ethereal sulphate of isomer A was isolated from the glucuronide gum as its Safranine bluish salt (Dodgson, Rose & Spencer, 1955) and was converted by cation exchange into potassium 3-*tert.*-butyl-4-methoxyphenyl sulphate; both salts were identical with synthetic specimens. Compound H was formulated as a 2 (or 3)-*tert.*-butyl-4-hydroxyphenyl sulphate, since mild acid hydrolysis of a chromatographically pure specimen yielded only SO_4^{2-} ions and *tert.*-butylquinol, identified spectroscopically and chromatographically. The glucuronide of isomer B was isolated via its barium salt (Dacre *et al.* 1956) from the glucuronide gum. No ethereal sulphate of isomer B was isolated, although its Safranine bluish salt was readily prepared. No other metabolites of isomers A and B were detected.

EXPERIMENTAL

Melting points are uncorrected.

Examination of urine for metabolites

Metabolism of butylated hydroxyanisole. Urine (860 ml.) from Expt. 18, total dose 15·5 g., was clarified by centrifuging and the sediment reserved; the liquid was extracted with ether (2 × 800 ml.) by gentle shaking for 2 hr., and the ether extract reserved. Glucuronide gums were obtained in the usual way by precipitation with lead acetate soln. at pH 4·5 and 9·5 (Kamil *et al.* 1951).

Sediment. The dry sediment yielded an ether-soluble fraction (0·25 g.), which two-dimensional chromatography showed to be isomer A mixed with a trace of isomer B.

Ether extract. The semi-crystalline residue yielded on distillation at 35 mm. Hg and 150–155° a crystalline solid (0·41 g.), shown chromatographically to be isomer B [R_F in solvent III (2), 0·90] with a trace of isomer A [R_F in solvent III (2), 0·85].

Glucuronide gum (pH 4·5). The aqueous solution (500 ml.) mixed with saturated barium acetate solution (8 ml.) was slowly concentrated *in vacuo*. An inorganic precipitate was discarded. The filtrate yielded a gum which was redissolved, freed from Ba^{2+} ions with CO_2, the filtrate evaporated and the residue extracted with ethanol and the ethanol extract concentrated to a gum (4·9 g.). This was shown, by hydrolysis of 0·017 g. with β-glucuronidase, and chromatography of the ether extract of the hydrolysate, to contain 0·26 g. of mixed isomer A and B glucuronides as butylated hydroxyanisole, and no glucuronides other than those normally found in this fraction. The total SO_4^{2-} ion content of the gum, measured gravimetrically, was equivalent to 0·03 g. of ethereal sulphates as butylated hydroxyanisole.

Table 4. *Urinary excretion of butylated hydroxyanisole and its metabolites by rats after a single dose*

Results are expressed as % of dose fed. Each rat in a group of four (Expts. 12, 13) or eight (Expts. 8–11) received butylated hydroxyanisole by gastric tube in diluted pea soup. Analyses were performed on the pooled daily output of all rats in a group, collected until no further metabolites were detectable. Superscripts indicate the number of groups in an experiment. Butylated hydroxyanisole was estimated chromatographically, and glucuronides chromatographically after enzymic hydrolysis, both as isomer B. Ethereal sulphates were estimated gravimetrically.

Expt. no.	Dose (mg./rat)	Dose (g./kg.)	Butylated hydroxyanisole in medium (%)	Free butylated hydroxyanisole	Period of excretion (days)	Glucuronide	Period of excretion (days)	Ethereal sulphate	Period of excretion (days)	Total accounted for
8[1]	31·5	0·1	10	4	7–8	75	5–6	12	3–4	91
9[1]	13·5	0·05	10	5	3–4	69	5–6	10	2–3	84
10[1]	7·6	0·025	1	8	3–4	92	4–5	Not detectable	.	100
11[1]	3·7	0·01	1	14	4–5	.	.	Not detectable	.	.
12[2]	3·6, 3·9	0·01	1	7, 12	2–3	70, 74	2–3	Not detectable	.	81, 82
13[2]	0·82, 0·85	0·002	0·35	9·5, 11	2–3	72*	2–3	Not detectable	.	82

* Estimated on the pooled daily output of both groups.

Table 5. *Urinary excretion of butylated hydroxyanisole and metabolites during successive doses of 0·5 g./kg.*

Results are expressed as % of dose fed. Compounds were injected by gastric tube: (*a*) as a 50% (w/v) solution in propylene glycol; (*b*) as a 10% (w/v) suspension in diluted pea soup; (*c*) as a 50% (w/v) solution in corn oil. Each rat of a group of rats was given successive daily doses. Urines were collected for the duration of dosing and pooled. Unless specified otherwise, sulphates were measured gravimetrically and glucuronides by the modified naphtharesorcinol method. Superscript numerals indicate the number of doses per rat.

Compound	Expt. no.	Total average dose/rat (g.)	Medium	No. of rats	Free butylated hydroxy-anisole	Gluc-uronide	Ethereal sulphate	Total accounted for
	14	0·81	*b*	4^{8}	6	47	23*	76
Isomer A	15	0·77	*c*	6^{5}	5	24†	30	59
					1‡	10‡	5‡	16‡
	16	0·52	*a*	3^{8}	7	43	6*	56
Isomer B	17	1·05	*b*	6^{4}	2	70†	3	75
Butylated hydroxyanisole	18	1·94	*b*	8^{10}	3	46	3	52

* Estimated by Sperber's method (Bray & Thorpe, 1954). † Estimated enzymically.
‡ Excreted in 12 days after the last dose in Expt. 15.

Glucuronide gum (pH 9·5). The aqueous solution (750 ml.), mixed with saturated barium acetate solution (10 ml.) and adjusted to pH 10·0, was slowly concentrated *in vacuo*. Needles of the barium salt of the glucuronide of isomer B (10·2 g.) separated and were converted into 2-*tert.*-butyl-4-methoxyphenyl-β-glucosiduronic acid, m.p. 168–169°, by the method of Dacre *et al.* (1956) (Found: C, 57·5; H, 6·8; Calc. for $C_{17}H_{24}O_8$: C, 57·3; H, 6·8%). The i.r. spectrum had a strong band at 5·75 μ (carboxyl C:O) and enhanced absorption, compared with isomer B, at 3 μ (OH).

The supernatant of the glucuronide precipitation was freed from Ba^{2+} ions with CO_2, and the filtrate gave on evaporation *in vacuo* a clear-yellow gum (19·4 g.), shown by enzymic hydrolysis to contain 0·39 g. of unprecipitated glucuronides of butylated hydroxyanisole. The gum was twice extracted with hot ethanol (100 ml.) and since the ethanol-insoluble portion contained no metabolites it was discarded. Comparison of the ethanol-soluble gum with the corresponding gum from control rat urine by enzymic hydrolysis and two-dimensional chromatography of the ether extract of the hydrolysate revealed that the only glucuronides not normally present were those of isomer A and B.

Residual urine. After the removal of lead with H_2S, the combined filtrate and washings (1·5 l.) were concentrated *in vacuo* to low volume, stirred for 1 hr. with stearic acid-deactivated charcoal (15 g.; Asatoor & Dalgliesh, 1956), the charcoal was collected and the filtrate, which was chromatographically free from detectable metabolites of butylated hydroxyanisole, discarded. The substances adsorbed by the washed charcoal were removed by elution with 1 l. each of (successively) aq. 7·2% (v/v) phenol, aq. 6N-NH_3 soln.–aq. 7·2% (v/v) phenol (1:5, v/v) and 6N-HCl–aq. 7·2% (v/v) phenol (1:5, v/v). Paper-chromatographic examination of the phenol-free residues from the eluates revealed no substances, other than a trace of compound H, which were not present in the corresponding fractions of control urine.

Metabolism of isomer A. (i) *Isolation of the glucuronide as its amide.* Clarified urine (260 ml.) from Expt. 14 was shown chromatographically to contain compound H and isomer A; its ether extract contained isomer A, the impurity *p*-methoxyphenol and normally occurring phenols. The pH 4·5 glucuronide gum yielded no combined isomer A, and enzymic hydrolysis showed the pH 9·5 glucuronide gum (1·9 g.) to contain the glucuronides of isomer A and, in trace amounts, of *p*-methoxyphenol. Solvent extraction of this gum, partition chromatography, methylation and acetylation yielded intractable products. It was extracted with cold propan-2-ol (3 × 350 ml.), the slightly dextrorotatory extract was evaporated to an amorphous solid which was methylated and acetylated, the dried product extracted with boiling hexane (200 ml.) and the extract chromatographed on a column of acid-washed alumina prepared from a hexane slurry. Fractions (100 ml.) were selected by extinction at 280 mμ; those eluted in order by benzene–$CHCl_3$ (2:3, v/v), benzene–$CHCl_3$ (1:4, v/v) and $CHCl_3$ were combined and evaporated. The residue was dissolved in methanol (20 ml.), the chilled solution was saturated with anhydrous NH_3 and kept for 24 hr. at 5°. The methanol was removed and the residue crystallized from water to yield white prisms of 3-tert.-*butyl-4-methoxyphenyl-β-glucosiduronamide* (8·5 mg.), m.p. 237·0–238·5° (decomp.) not depressed on admixture with a synthetic specimen (Found: C, 57·1; H, 7·5. $C_{17}H_{25}O_7N$ requires C, 57·4; H, 7·1%); natural and synthetic materials had identical u.v. and i.r. absorptions. We were unable to obtain the barium salt of this glucuronide prepared by Dacre *et al.* (1956).

(ii) *Isolation of the ethereal sulphate.* The copious precipitate of lead salts, obtained at pH 9·5 from clarified urine (510 ml.) from Expt. 15, was suspended in water and lead was removed as PbS; the solution was partly concentrated at 35° *in vacuo* to remove H_2S, adjusted to pH 9·5 with K_2CO_3 and evaporated *in vacuo* at 40° to a gum. This was extracted with cold ethanol (500 ml.), and the ethanol extract yielded a clear gum, from which, on addition of saturated Safranine bluish solution (10 ml.), red needles separated (1·26 g.), which crystallized from aq. ethanol. 3-*Amino-7-dimethylamino-5-phenylphenazinium* 3-tert.-*butyl-4-methoxyphenyl sulphate* (0·42 g.) had m.p. 230–235° (Found: C, 64·4; H, 5·9; N, 10·4. $C_{31}H_{34}O_5N_4S$ requires C, 64·8; H, 6·0; N, 9·7%); i.r. and u.v. spectra were identical with those of synthetic material (m.p. 233–235°), also R_f (0·87) in solvent I. The Safranine salt (400 mg.) was shaken for 2 hr. with Dowex-50 (K^+ form), and the solution was filtered and concentrated *in vacuo*; the residue, recrystal-

red from ethanol, yielded *potassium* 3-tert.-*butyl*-4-*methoxyphenyl sulphate* (74 mg., 37%). The plates, recrystallized from propan-2-ol, had an indeterminate m.p. (Found: C, 43·8; H, 5·2; K, 13·1. $C_{11}H_{15}O_5SK$ requires C, 44·3; H, 5·1; K, 13·1%); i.r. and u.v. spectra, chemical and chromatographic properties were identical with those of synthetic material.

Properties and attempted isolation of compound H. Compound H was detected on chromatograms in solvent I of urine from all rats dosed with isomer A. Its absence from urine of rats dosed with *p*-methoxyphenol showed that it was not a metabolite of this impurity in isomer A. Compound H remained in urine after extraction with ether and after precipitation of glucuronides. Lead-free urine thus treated from Expt. 15 was concentrated to a stiff gum which was macerated under hot acetone (1 l.) and the extract evaporated to a gum; the acetone-insoluble portion was discarded. The gum was stirred in water (100 ml.) for 1 hr. with stearic acid-deactivated charcoal, when compound H was completely adsorbed. The adsorbed substances were eluted with aq. 7·2% (v/v) phenol and aq. 6N-NH_3 soln.–aq. 7·2% (v/v) phenol (1:5, v/v), and the eluates on evaporation *in vacuo* and removal of phenol yielded gummy residues which were combined (0·52 g.), streaked on eight sheets of Whatman no. 4 paper, 18·5 in. × 22 in., and chromatographed in solvent I. Compound H, located by co-chromatography, was eluted from the required cut-out zones with solvent I and the eluate was evaporated to a brown gum, which was washed with benzene and propan-2-ol. The light-brown semi-crystalline residue (10 mg.) had the reactions shown in Table 1 and the light-absorption maxima shown in Table 2. Compound H gave no Fe^{3+} ion ninhydrin reactions on paper and no naphtharesorcinol reaction in solution. The i.r. spectrum had bands at 3·0 and 9·5μ (S:O). Gentle warming with 3N-HCl liberated SO_4^{2-} ions and an ether-extractable phenol, which had the chromatographic properties (Table 1) and the u.v. spectrum of tert.-butylquinol. Both the liberated phenol and *tert.*-butylquinol gave in aq. ethanol the same pale-yellow Fe^{3+} ion colour, which deepened on addition of 1 drop of 2N-NaOH. Compound H is probably a 2 (or 3)-*tert.*-butyl-4-hydroxyphenyl sulphate, derived from isomer A by *O*-demethylation and subsequent ethereal sulphate conjugation of either hydroxyl, or by *O*-demethylation of the ethereal sulphate of isomer A.

Metabolism of isomer B. Continuous ether extraction of the urine (120 ml.) from Expt. 16 yielded isomer B, b.p. 150–155° at 35 mm. Hg, identical with the material fed. The glucuronide gum yielded 2-*tert.*-butyl-4-methoxyphenyl-β-glucosiduronic acid (0·36 g.) as before, m.p. and mixed m.p. 168–169°. Exhaustive chromatographic examination of the enzymic hydrolysates of the glucuronide gum and of the glucuronide-free urine revealed no further metabolites of isomer B. No ethereal sulphate Safranine bluish salt was obtained from the glucuronide gum.

Synthesis of ethereal sulphates

These were prepared by the method of Feigenbaum & Neuberg (1941).

Potassium 3-tert.-*butyl*-4-*methoxyphenyl sulphate.* A mixture of carbon disulphide (20 ml.), diethylaniline (5 ml.) and chlorosulphonic acid (2 g.) was slowly added with stirring to a chilled solution of isomer A (3 g.) in carbon disulphide (20 ml.) and diethylaniline (2 ml.). After 3 hr., an excess of aq. 50% (w/v) KOH was added slowly, followed by ether (200 ml.); the precipitate was triturated with ether (200 ml.) and extracted with hot propan-2-ol. The cooled extract yielded colourless prisms (2·4 g., 48%) of indeterminate m.p. (Found: C, 44·2; H, 5·0; S, 10·8; OCH_3, 11·0; K, 13·1. $C_{11}H_{15}O_5SK$ requires S, 10·7; OCH_3, 10·4%) having absorption at 9·42μ (S:O). It decomposed at 65° *in vacuo* and above 120°. On gentle warming of a portion with 2N-HCl, isomer A was detected chromatographically in the ether extract of the solution and the solution also gave a strong SO_4^{2-} ion reaction. An insoluble lead salt was formed at pH 9·5. Addition of saturated aq. Safranine bluish solution to aq. 10% (w/v) potassium 3-*tert.*-butyl-4-methoxyphenyl sulphate yielded lustrous green–red needles, recrystallized from aq. ethanol. 3-*Amino*-7-*dimethylamino*-5-*phenylphenazinium* 3-tert.-*butyl*-4-*methoxyphenyl sulphate* had m.p. 233–235° (Found: C, 64·8, 64·7; H, 5·8; 6·0; N, 9·9; S, 5·3. $C_{31}H_{34}O_5N_4S$ requires S, 5·6%), R_F in solvent I, 0·87. It had $\lambda_{max.}$ 229 (log $\epsilon_{max.}$ 4·28) and 274 mμ (log ε, 4·69). The Safranine salt (50 mg.) in water (10 ml.) was shaken for 1 hr. with Dowex-50 (K^+ form) (5 g.), the solution was filtered, evaporated to dryness *in vacuo* and the residue, recrystallized from propan-2-ol, yielded potassium 3-*tert.*-butyl-4-methoxyphenyl sulphate (16·4 mg., 65%).

Potassium 2-tert.-*butyl*-4-*methoxyphenyl sulphate.* This, prepared as described above from isomer B in 50% yield, recrystallized from propan-2-ol as prisms, had an indeterminate m.p. (Found: C, 44·3; H, 5·2; S, 10·7; OCH_3, 10·1; K, 13·2. $C_{11}H_{15}O_5SK$ requires C, 44·3; H, 5·1; S, 10·7; OCH_3 10·4; K, 13·1%) and strong absorption at 9·45μ. Its chemical properties were similar to those of the ethereal sulphate of isomer A. 3-*Amino*-7-*dimethylamino*-5-*phenylphenazinium* 2-tert.-*butyl*-4-*methoxyphenyl sulphate*, obtained as before, gave red needles from aq. ethanol, m.p. 216–218° (Found: C, 63·8; H, 6·1. $C_{31}H_{34}O_5N_4S$, 0·5 H_2O requires C, 63·8; H, 6·0%) and had $\lambda_{max.}$ 229 (log $\epsilon_{max.}$ 4·28) and 273 mμ (log $\epsilon_{max.}$ 4·69).

Synthesis of derivatives of the glucuronide of isomer A

Methyl (*tri*-O-*acetyl*-3-tert.-*butyl*-4-*methoxyphenyl*-β-*glucosid*)*uronate.* This was prepared by the procedure of Bollenback, Long, Benjamin & Lindquist (1955). Methyl-(tetra-*O*-acetyl-β-glucopyranosid)uronate (5 g.), isomer A (5 g.) and freshly fused $ZnCl_2$ (1 g.), dissolved in 20 ml. of acetic anhydride–acetic acid (1:20, v/v), were heated at 100° for 20 min., excess of solvent was removed *in vacuo* at 100° for 20 min., the residue was dissolved in benzene (100 ml.) and the solution washed with 2N-NaOH (2 × 100 ml.) and water (100 ml.), dried over $MgSO_4$ and concentrated *in vacuo*. A semi-crystalline residue resulted after several hours at 1·5 mm. Hg, and after trituration with hexane colourless needles were collected and recrystallized from hexane. *Methyl* (*tri*-O-*acetyl* 3-tert.-*butyl*-4-*methoxyphenyl*-β-*glucosid*)*uronate* (1·6 g.) had m.p. 122–123°, $[\alpha]_D^{24}$ −25·1° in $CHCl_3$ (*c*, 16·7) (Found: C, 57·6; H, 6·7; OCH_3, 12·9. $C_{21}H_{32}O_{11}$ requires C, 58·0; H, 6·5; OCH_3, 12·5%). Chromatographic treatment of the combined mother liquors on a column of acid-washed alumina yielded a further 600 mg. of product, m.p. 118–120·5° (total yield 15%). Longer heating of the reactants (90 min. at 100°) yielded, on chromatography of the product, needles of 3-tert.-*butyl*-4-*methoxyphenyl acetate* (4·5 g.),

m.p. 56–58°, identical with that prepared by acetylation of isomer A in pyridine and acetic anhydride, m.p. 58·0–58·5° (Found: C, 70·2; H, 8·5; OCH_3, 13·3. $C_{13}H_{18}O_3$ requires C, 70·2; H, 8·2; OCH_3, 13·9 %) and yellow needles (0·65 g.) of the anomeric *methyl (tri-O-acetyl-3-tert.-butyl-4-methoxyphenyl-α-glucosid)uronate*, m.p. 144–146°, $[\alpha]_D^{24}$ + 127° in $CHCl_3$ (*c*, 9·1) (Found: C, 58·4; H, 6·5 %), but no β-anomer. Anhydrous NH_3 was passed for 0·5 hr. into a chilled solution in methanol (30 ml.) of the β-anomer (300 mg.), the solution was chilled for 3 days, the solvent removed and the residue crystallized from water to yield needles (135 mg.) of *3-tert.-butyl-4-methoxyphenyl-β-glucosiduronamide*, m.p. 238·5° (decomp.), $[\alpha]_D^{24}$ – 188° in ethanol (*c*, 0·9) (Found: C, 57·0; H, 7·3; N, 4·3. $C_{17}H_{25}O_7N$ requires N, 3·9 %). The i.r. spectrum showed strong bands at 2·84, 2·93 and 5·98 μ and the u.v. spectrum had $\lambda_{max.}$ 226 (log ε 3·93) and 284 mμ (log ε, 3·43).

Attempted preparation of the glucuronide of isomer A. Hydrolysis of the methyl tri-*O*-acetyl-β-anomer with $Ba(OH)_2$ solution or aq. methanolic suspensions of $Ba(OH)_2$ yielded amorphous products. Catalytic deacetylation of 1·34 g. with sodium (0·05 g.) in methanol (300 ml.) yielded, after 6 days, a semi-crystalline mass on evaporation *in vacuo*, which was purified through its lead salt or over Dowex-2 (H^+ form) to give a residue which separated from propan-2-ol as a glass (270 mg.), $[\alpha]_D^{24}$ – 46·5° in water (*c*, 3·4). The product gave a strong positive naphtharesorcinol reaction and had pH 3·7 in 0·1 % solution. Hydrolysis with β-glucuronidase (10^4 Fishman units) of 2·8 μmoles gave 2·45 μmoles (84 %) of isomer A, estimated chromatographically, and u.v. (Table 2) and i.r. spectra (bands at 3·0, 5·8 μ) were closely similar to those of the glucuronide of isomer B. This was the purest specimen prepared of the glucuronide of isomer A.

DISCUSSION

The metabolism of butylated hydroxyanisole in the rat resembles that reported for the rabbit for doses of 0·13–0·55 g./kg. (Dacre *et al.* 1956). At all doses studied in the rat butylated hydroxyanisole and its component isomers, on ingestion, are absorbed and readily metabolized. The possible metabolic pathways for phenols and substances analogous to butylated hydroxyanisole (Williams, 1949; Brodie, Gillette & LaDu, 1958) suggest that 4-*O*-conjugation, *O*-demethylation, hydroxylation of the phenyl ring and side-chain oxidation ($-CH_3 \rightarrow CH_2 \cdot OH$) might be involved in metabolism of butylated hydroxyanisole. However, the main metabolites are 4-*O*-conjugates, i.e. the *O*-sulphate and *O*-glucuronide of isomer A and the *O*-glucuronide of isomer *B*. The only other metabolic pathway is the probable *O*-demethylation of isomer A, which yields compound H. In the rabbit both isomers are metabolized largely by 4-*O*-glucuronide formation (Dacre *et al.* 1956).

The conjugation of isomer B appears to differ from that predictable from steric considerations. The 4-hydroxyl group in isomer B is clearly crowded by the 3-*tert.*-butyl group, such that conjugation with the ethereal sulphate group would seem preferable to conjugation with the bulkier glucuronide group (Fisher–Taylor–Hirschfelder Models, Fisher Scientific Co., Pittsburgh, Pa., U.S.A.). However, isomer B forms mostly an *O*-glucuronide. Isomer A does not appear to possess steric restrictions on conjugation, and forms mainly an ethereal sulphate, *O*-glucuronide formation apparently becoming significant only when the sulphate pool is depleted by repeated large doses.

In the single-dose studies, at the lowest dose (2 mg. of butylated hydroxyanisole/kg.) the background absorption of chromatograms in the zones examined for isomer B, owing to the excretion of normal metabolites, is no longer negligible. Thus the apparent glucuronide excretion (enzymic method) of controls, expressed as isomer B, amounts to 18 % (0–39 %)/day of a dose of 0·82 mg. of butylated hydroxyanisole/rat. The apparent excretion of free isomer B by controls amounts to 6 % (0–13·5 %)/day of the same dose. In Expt. 13, however, actual control values, determined on the day before dosing, were glucuronide, 0 %, and free isomer B, 3·8–4·0 %, of the dose, and were assumed to be unaltered during urine collection. In other experiments at this dose, not reported, background absorption was too high to enable a reliable estimation of dose excretion to be made. This dose is clearly a lower limit for the method.

Over the range studied, the excretion of unconjugated butylated hydroxyanisole decreased from 11 % (7–14 %) for a 10 mg./kg. dose to 3–7 % for a 400 mg./kg. dose. With 0·3 mg./kg. in dogs H. C. Hodge & D. W. Fassett (unpublished) found negligible tissue storage, and in the present study over-all recoveries of a single dose remained uniformly high (82–100 %) at all doses studied. The slightly higher proportion of unconjugated butylated hydroxyanisole excreted at the lowest doses is therefore unlikely to be associated with any increase in tissue storage.

The above study approaches closer to human-usage doses than does any previous study on antioxidants, and, although the problem of studying the human-usage dose remains, extrapolation to that amount seems justified. No evidence has so far been presented which suggests that butylated hydroxyanisole is not largely absorbed and largely excreted at these very low doses.

SUMMARY

1. The metabolism of butylated hydroxyanisole and 2- and 3-*tert.*-butyl-4-hydroxyanisole in the rat is studied with particular emphasis on the fate of butylated hydroxyanisole at low doses.

2. Increased urinary glucuronide and ethereal sulphate excretions during 5 days after dosing with

0·4 g. of butylated hydroxyanisole/kg. account for 61–82 and 11–16 % respectively of the dose, and 5 % of the dose is excreted, unchanged.

3. For single oral doses of 0·002, 0·01, 0·025, 0·05 and 0·1 g. of butylated hydroxyanisole/kg., overall recoveries of the dose from the urine were 81–100 %. Glucuronide excretion accounts for 69–92 % of the dose, and a slight increase in excretion of unchanged butylated hydroxyanisole occurs at the lowest doses.

4. For single oral doses of 0·4 g. of 2-*tert.*-butyl-4-hydroxyanisole 72 % of the dose is excreted as ethereal sulphate in 5 days. With 3-*tert.*-butyl-4-hydroxyanisole 57–71 % of the dose is excreted as glucuronide in 5 days.

5. The administration of several successive daily doses of 0·5 g. of butylated hydroxyanisole/kg., and of 3-*tert.*-butyl-4-hydroxyanisole, decreases the percentage recovery of conjugates and the proportion of ethereal sulphate excreted during dosing; repeated doses of 0·5 g. of 2-*tert.*-butyl-4-hydroxyanisole/kg. cause a considerable increase in glucuronide and a decrease in ethereal sulphate conjugation of the dose.

6. The isolation of the major metabolites, the probable formation of a demethylation product of 2-*tert.*-butyl-4-hydroxyanisole as a minor metabolite, the synthesis of the ethereal sulphates of both isomers and the synthesis of the glucuronamide of 2-*tert.*-butyl-4-hydroxyanisole are described.

We wish to thank Dr C. J. Terhaar, and the staff of the Toxicology Laboratory, for the care and dosing of experimental animals; Mr Stuart Foster of the Industrial Laboratory for infrared and ultraviolet spectra; and Mr D. F. Ketchum of the Microanalytical Laboratory for microanalyses.

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SEE QUESTION 5 - BOX 66

6th Report of the Joint FAO/WHO Expert Committee on Food Additives. Geneva, 1962 (WHO Technical Report Series No. 228)

COMPANY CODE: [redacted]

FFC No.: 0032

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Further work considered desirable

If the use of boric acid as a food additive is continued, long-term studies should be made, with particular attention to the possibility of cumulation.

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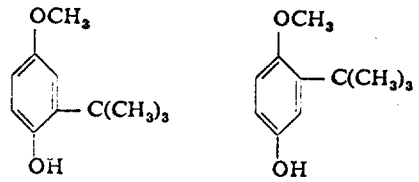
BUTYLATED HYDROXYANISOLE

Chemical name A mixture of 3- and 2-*tert.*-butyl-4-hydroxyanisole

Synonym BHA

Empirical formula $C_{11}H_{16}O_2$

Structural formula



3-isomer 2-isomer

Molecular weight 180.25

Definition Butylated hydroxyanisole is a mixture of 85% or more of 3-*tert.*-butyl-hydroxyanisole and 15% or less of 2-*tert.*-butyl-hydroxyanisole[1] and should contain not less than 98.5% of $C_{11}H_{16}O_2$.

Description A white or slightly yellow, waxy crystalline solid with an aromatic odour. Insoluble in water but soluble in fats. 1 g is soluble in 4 ml of ethanol.

Use As an antioxidant for fats and oils and in packaging material for fat-containing foods; its activity is enhanced in combination with other phenolic antioxidants and synergists.

Biological Data

Acute toxicity

Animal	Route	LD_{50} (mg/kg body weight)	References
Mouse	oral	2000	2,3
Rat	oral	2200 to > 5000	2,3

Short-term studies

Rat

No effect on potassium excretion, as described below for the rabbit, was observed in the rat.[4]

Groups of 7 recently weaned rats were fed for six months on rations containing 0%, 0.5%, 1%, 2% and 3% of BHA. The rats at the 3% level did not eat enough to gain weight and were put on to the 2% diet for a time, then returned to 3%. Even at the 2% level, food consumption was not optimal. Histopathological examination revealed no pathological condition attributable to BHA.[5]

Combinations of BHA with other food additives, such as chlorine dioxide, sodium propionate, propyl gallate, or polyoxyethylene-8-stearate, at fifty times the normal levels of use in bread, had no deleterious effects when they were fed in bread to groups of 26 rats for a period of 32 weeks. The treated bread formed 75% of the animals' diet. The daily dosage levels of BHA were from 3.3-7.0 mg/kg body weight.[6, 7]

Rabbit

In rabbits, a dose of 1 g given daily for 5 to 6 days by stomach tube caused a tenfold increase in sodium excretion and a 20% increase in potassium excretion in the urine. Extracellular fluid volume fell, and this prevented any marked change in the plasma sodium level. The serum potassium fell after 5 days' treatment and potassium was being replaced by sodium in muscle cells. In heart muscle the changes occurred later than in skeletal muscle and were less marked. The antioxidant may have a direct effect on the kidney: the adrenal cortex showed changes in the *zona glomerulosa* and there was increased excretion of aldosterone in the urine, associated with the sodium and potassium loss.[8]

Dog

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Long-term studies

Rat

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Dog

When BHA was fed to dogs at dose levels of 0, 0.3, 30 and 100 mg/kg body weight for one year, no ill effects were observed. Renal function, haematology and histopathology of the main tissues were normal. Organ weights were within normal limits and there was no demonstrable storage of BHA. The urine did not contain a demonstrable increase of reducing substances, even when 100 mg/kg body weight of BHA was fed. Groups of 3 dogs were used at each dose level for these experiments.[9]

Long-term studies

Rat

Groups of 15 or more newly weaned rats were placed on diets containing 0%, 0.05%, 0.5% and 1% BHA in lard (0%, 0.003%, 0.03% and 0.06% of the total diet) for 22 months. Weight gain was comparable in all groups. Reproduction was normal, and young rats kept on the same ration grew normally. Number, size, weight, weight gain and mortality of the litters were comparable for animals of all groups. After one year on test, the colony suffered from an infectious respiratory disease and many died. There was no significant difference in mortality among the groups. After 22 months, the remaining animals were killed; histopathological examination revealed no changes attributable to the antioxidant.[5]

A similar series of tests was undertaken, with an additional group on a diet of 2% BHA in lard (0.12% of the total diet). There were 17 rats in each group. After 21 months, the survivors were killed. Histopathological examination revealed no significant differences compared with the control animals. The rate of gain in weight during the growing period was unchanged, and all rats appeared normal in every respect.[5]

In another rat feeding test carried out over a period of 2 years on groups of 40 rats, there was a small reduction in the mature weight and an increase in relative liver weight in some cases with the highest level of BHA used (0.5% of the diet), but there were no effects on any of the following: the reproductive cycle; histology of the spleen, kidney, liver, or skin; ratio of weight of heart, spleen, or kidneys to total body weight; mortality. The toxicity of BHA was not affected by the dietary fat load.[10]

Biochemical aspects

BHA was absorbed from the gastrointestinal tract, and there was some evidence that the feeding of amounts 100-500 times the levels generally permitted in fats for human consumption (in the USA 200 mg/kg fat) caused deposition in depot fat, the stability of which was thereby increased.[11] However, there was no evidence of cumulation in other tissues.[1, 2, 9] In the rabbit, BHA was conjugated mainly with glucuronic acid or sulfuric

acid;[12] a small amount of unchanged BHA was excreted in the urine. In rats the 2-*tert*.-butyl isomer was chiefly excreted as glucuronide, while the 3-*tert*.-butyl isomer was excreted mainly as ethereal sulfate.[1] Thus, these animals effectively detoxicated BHA. The changes described occurred in the liver. No evidence has been found to suggest that BHA produces any adverse biochemical or metabolic effect in the animal body.[4]

Comment on experimental studies reported

Most of the studies reported have been carried out in rats and there is little work on other species. Negative results in groups of 3 dogs[9] fed for one year at each dose level are of limited use for evaluation. The metabolic studies have been done mainly in rabbits; differences between the rabbit and rat have been reported.

Long-term studies in rats have been carried out by several groups of workers. Some reservations can be made about each of these studies. However, taken together, the two long-term studies in rats[5, 10] provide a basis for evaluation. It is presumed that man metabolizes butylated hydroxyanisole similarly to the rat.

Evaluation

Level causing no significant toxicological effect in the rat

From consideration of the long-term studies in rats, the level causing no toxicological effect on feeding over a period approximating to the life span is 0.5% of the diet. An inconstant effect on relative liver weight and body weight gain in one series is disregarded.

0.5% (=5000 p.p.m.) in the diet is equivalent to 250 mg/kg body weight per day.

Estimate of acceptable daily intakes for man

	mg/kg body weight
Unconditional acceptance	0-0.5
Conditional acceptance	0.5-2.0

Comment

For levels of use in the conditional dosage zone attention should be paid to the presence of other phenolic antioxidants in the diet or environmental conditions involving significant exposure to phenolic substances.

Further work considered desirable

1. Long-term studies in a species other than the rat, with careful evaluation of tumour incidence.
2. Biochemical studies in human subjects.

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Chemical names

Synonym

Empirical formula

Structural formula

Molecular weight

Definition

Description

Use

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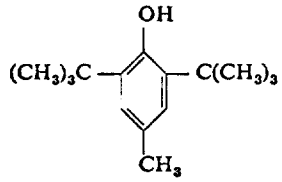
BUTYLATED HYDROXYTOLUENE

Chemical names	2 : 6-di-*tert*.-butyl-*p*-cresol ; 4-methyl-2 : 6-ditertiary-butylphenol
Synonym	BHT
Empirical formula	$C_{15}H_{24}O$
Structural formula	
Molecular weight	220.36
Definition	Butylated hydroxytoluene should contain not less than 99.0% of $C_{15}H_{24}O$.
Description	A white, crystalline, odourless solid. It is insoluble in water but soluble in fats; 1 g is soluble in 4 ml of ethanol.
Use	As an antioxidant for fats and oils or in packaging material for fat-containing foods. Its activity is enhanced in combination with other antioxidants and synergists.

Toxicologic Characteristics of Phenol Antioxidants of Alimentary Fats (Acute and Subacute Experiments)

I. A. Karplyuk

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Fats, which represent a valuable nutritional product, are subject to rapid spoilage, which, basically, results from oxidizing processes. Oxidation, at low temperatures, slows down somewhat, but it is not discontinued; for this reason, even in the refrigerator, fats may not be preserved for no longer than half a year. In the oxidation process, fats lose their taste (flavor) and nutritive properties and may produce a harmful action (I. Smorodintsev, 1947; Burr and Barues, 1943; Johnson, Perkins, Sugal and Kummerow, 1957, and others).

It has not yet been clarified, definitively, whether the very products of fat oxidation (peroxides, polymeric products) produce a direct harmful effect, or whether, during the oxidation of fats (during rancidification), there is an oxidation and inactivation of such important constituent parts of the diet as vitamins A, D, E, K, pantothenic acid, pyridoxine, biotin, ascorbic acid and vitally necessary unsaturated fatty acids (linoleic, linolenic, and arachidonic). Pathological phenomena, which developed during the feeding of rats with a rancidified experimental diet, are explained by a shortage of these nutritive substances in the diet (Quackenbusch, 1945; Greenberg and Frazer, 1953, Witting, Hishida, Johnson and Kummerow, 1957).

Data also exist with reference to the fact that thoroughly (deeply) oxidized fats possess a carcinogenic activity (Lavik and Baumann, 1941, 1943; Roffo, 1938) The spectrum of thermally oxidized fat has a resemblance to the spectrum of carcinogenic derivatives of phenanthrene (Roffo, 1938).

One of the effective methods of keeping fats from oxidation is the introductio of antioxidants in the fats. The greatest interest is being shown in phenolic

antioxidants as being the most effective: butyloxyanisol, butyloxytoluol and gallic acid esters. In a number of countries, these antioxidants have found wide usage 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 have proposed the introduction of butyloxyanisol (BNA), butyloxytoluol (BNT) and propylgallate (PG) into rendered fats and vegetable oils. It is recommended that the antioxidants be introduced in an amount of 0.01 - 0.02%. With such an amount of butyloxyanisol or butyloxytoluol, the fats increase their stability by 3 - 5 times, and in the case of adding gallates -- up to 10 times (N. Emanuel', D. Knorre, Yu. Lyaskovskaya and V. Piul'skaya, 1955, 1958). These antioxidants, in the indicated amounts, do not bring about any organoleptic changes in the fat.

When these preparations were proposed for their application in the food industry, a question wich regard to their toxicity arose. A study of the toxicity of butyloxyanisol was taken up by Lehmann, Fitzugh, Helson and Woodard (1951), Wilder Ostby and Gregory (1956) and others. Takata, Nagata (1952), Deichmann, Clemmer, Rakoczy and Bianchine (1955) made a study of butyloxytoluol. The toxicity of the gallates was determined by Boehm and Williams (1943), Orten, Knyper and Smith (1948), Allen and De Eds (1951), Roy (1953), Sporn and Peretianu (1956). By the papers (studies) of the above-enumerated authors there were established the LD_{50} of these preparations for mice, rats, rabbits, guinea pigs, and cats; at the same time, the method of establishing this dose is different in the case of each author: some established it by subcutaneous administration, and others -- by an intraperitoneal one, and still others -- by an enteric one (enterally); solutions of various concentrations were administered. It is understandable that the results obtained differed from one other. In the case of a prolonged administration (1 - 3 months) of large doses of phenolic antioxidants (close to 1% of the weight of the feed), the authors noticed an impairment in the growth and development of the animals. Propylgallate, inthe case of such concentrations, led, in addition to this, to a diminution in the amount of blood hemoglobin and to patho-

logical phenomena with respect to the kidneys (the appearance of albumen in the urine). Butyloxyanisol, with a concentration of 0.25 gram for 1 kilogram of weight, in dogs, brought about degenerative changes in the parenchymatous tissue of the liver. The feeding of higher gallates (octyl, dodecyl) to rat females, in a concentration of 0.5% of the weight of the feed, brought about an impairment in lactation and manifested a harmful effect on the young generation. It should be noted that all the authors, in subacute experiments, made use of a very limited number of tests: the weight of the animals, a study of the blood, and pathomorphological studies of the organs. Only Deichmann and others (1955), in studying the toxicity of butyloxytoluol, determined the prothrombin time and measured the blood pressure. It seems to us that the amount of tests employed by the authors is insufficient in order to judge with respect to the fuctional state of the organism. In our experiments we made an attempt, to the broadest extent possible, to study the various sides of the organism's active life (life activity).

For the comparative toxicologic characteristics of the preparations under study (butyloxyanisol, butyloxytoluol, and propylgallate), acute experiments were set up on rats and mice. For the experiment were taken growing, healthy animals (males and females). Antioxidants were administered in the morning, on an empty stomach, in a solution of sunflower oil, directly into the esophagus from a syringe through a metallic probe, represented by an ordinary thick injection needle with an "olive" soldered on at the end. The animals that remained alive after the administration of the preparation stayed under observation over a period of 20 days. Various amounts of the preparations under study were administered to the animals (7 - 8 doses). Each dose was received by 8 rats and 10 mice.

By carrying out inoculations on the animals, in various doses, of the preparations under study, we established the toxicity of these substances from a minimal toxic dose to an absolutely fatal one. After that, having carried out an integration, according to Berens (N. S. Pravdin, 1947; V. S. Shadurskaya, 1953), which has been accepted in toxicology, we plotted a mortality curve, and according to it, determined the LD_{50} of all three antioxidants under study for rats and mice. The results of the experiments are set forth in Table 1.

((Table on pg. 25 of text))

Table 1

Results of Determining the Toxic and Fatal Doses of the Antioxidants Under Study

Antioxidants	Rats			Mice	
	minimal toxic dose in milligrams for 1 kilogram of weight	LD_{50} in milligrams for 1 kilogram of weight	LD_{100} in milligrams for 1 kilogram of weight	LD_{50} in milligrams for 1 kilogram of weight	LD_{100} in milligrams for 1 kilogram of weight
Butyloxyanisol (BNA)	1000	2900	4500	1250	3000
Butyloxytoluol (BNT)	500	2450	3500	2000	2500
Propylgallate (PG)	500	2600	3500	1700	2500

((text continued)): From the data obtained it may be seen that the toxicity of these preparations is very close with respect to one another and that phenolic antioxidants are comparatively low in toxicity.

Subacute experiments were conducted with the purpose of disclosing functional impairments under the influence of the preparations under study, in deliberately (admittedly) large, aggravated doses (in comparison with those that may find their way into a person's organism). In the event of the discovery of such deviations, attention was fixed precisely onthose tests in chronic experiments in the definitive solution of the problem concerned with the possibility of introducing phenol antioxidants into food fats. In subacute experiments, there were utilized four groups of rats: the first group received, in its diet, fat with BNA in a dose of 1/5 of LD_{50}, i.e., 600 milligrams for 1 kilogram of body weight; the second group received BNT, initially 1/5 of LD_{50}, i.e., 500 milligrams for 1 kilogram of weight; in the future, this dose was reduced to 250milligrams for 1 kilogram of body weight because of a poor consumption of feed in the case of a higher content of BNT; the third group received 1/5 of LD_{50} with respect to PG

— 500 milligrams for 1 kilogram of body weight; the fourth group was a control group.

Antioxidants were injected into rendered lard which was mixed in with the basic feed. The control group received pure lard. The rendered lard and the antioxidant preparations, which are necessary for the setting up of the experiments, were furnished to us by the All-Union Scientific-Research Institute of the Meat Industry.

Each rat was kept in an individual cage; this made it possible to keep track of the amount of the feed and fat, with antioxidants, which were consumed.

For the purpose of observing the functional state of the experimental (test) animals, we dwelt on (emphasized) the following indices.

1. Daily observation of the animals.

2. Weighing of the animals once very 10 days.

3. Carrying out a morphological examination of the blood, considering that it is known that phenol and its derivatives frequently produce a haematopoiesis (blood formation) pathology. In the morphological investigation, a count of the regular (prescribed) elements was made: of the erythrocytes, reticulocytes, thrombocyes, and leucocytes; a determination was made of the hemoglobin content an of the leucocytic formula.

4. Proceeding from the fact that the oxidizing processes lie at the basis of the majority of biochemical reactions in the organism, and that antioxidants, possibly, even in the live organism, manifest an effect on the oxidation processes an investigation was made of the activity of oxidizing enzymes, and to be precise, catalases and peroxidases. These enzymes were selected also for the reason that Tappel and Marr (1954), while studying in vitro the action of propylgallate and nordihydroguaiaretic acid (also that of a phenol antioxidant) on 14 enzymatic systems, discovered that they, in a concentration of $1 \times 10^{\text{minus } 4}$ M, inhibit the activity of a catalase and that of a peroxidase. The inhibiting action of phenolic compounds on a catalase was also shown by Goldacre and Galston (1953). The catalase was determined according to the method of Bakh and Zybkova and was expressed in the form of a catalase index. The peroxidase was determined from

the oxidation of indigocarmine and was expressed in the time (seconds) that was required for its oxidation.

5. An investigation was made of the cholinesterase activity of blood serum, which, in the opinion of physiologists (K. M. Bykov, 1949; Kh. S. Koshtoyants, 1946), is closely associated with the functional state of the nervous system.

6. An investigation was made of the prothrombin time (it depends on the amount of prothrombin that is synthesized in the liver). Clinical physicians acknowledge the value of this determination for the purpose of disclosing the functional impairments of the liver.

All the selected indices were verified in the animals prior to the experiment and following the administration of the antioxidants -- each 2 weeks. The animals were kept on the experiment from 68 to 82 days. At the end of the experimental period, all the animals were destroyed by decapitation. There were carried out a pathological-anatomical dissection and a histological investigation of the internal organs. In addition to a pathohistological examination, the liver was subjected to a chemical study; a determination was made of the content of dry substances, of the amount of glycogen, fat and lipoid phosphorus for the purpose of revailing any possible impairment of some aspects of the carbohydrate and fat metabolism in this organ.

During the entire experimental period, each experimental (test) animal received the following amount of preparations: BNA -- from 5 to 7 grams; BNT -- from 2 to 4 grams; PG -- from 4 to 6 grams.

No changes were observed in the behavior and outward appearance of the experimental (test) animals. Weighing of the animals indicated that prior to the administration of antioxidants, the animals in all groups added weight equally well. From the beginning, however, of introducing antioxidants into the diet, the weight of the experimental (test) animals started to lag behind the weight of the control (ones) considerably (Fig. 1). The lag in weight of the animal experimental (test) groups was especially pronounced in the first month of the experiment, and less -- in the second month.

In animals that received BNA and BNT in their diet there was disclosed a reduction in the activity of the blood catalase. In the control rats, at the end of the experimental period, the activity of the catalase comprised 9.5 - 13.5 milligrams of hydrogen peroxide; in the group with BNA -- 6.7 - 8.8 milligrams; in the group with BNT, by the 43rd day of the experiment -- 6.1 - 8.8 milligrams; by the 60th day, the activity of several increased but did not attain the catalase level of the control animals. Propylgallate did not bring about any changes in the catalase activity.

The blood of all three experimental (test) groups of animals indicated a reduction in the peroxidase activity (the amount of seconds devoted tothe oxidation of the substrate increased). The peroxidase activity of the blood in the control animals, during the period of the experiment, fluctuated within the range of 56 - 86 seconds, whereas in the group that received BNA, it comprised, by the 42nd day, 105 - 147 seconds, and by the end of the experiment -- 91 - 135 seconds. The group of animals that received BNT indicated a time of 83 - 116 seconds by the end of the experimental period, and the group, which had received propylgallate -- 84 - 110 seconds.

((Graph at the top left of pg. 27 of text)) ((to the left of this graph)): Weight (in grams); ((immediately underneath the same graph)): Weighing days; ((below that)): Fig. 1. Change in weight of rats in the course of the experimental period.

I -- weight of the control animals; II -- Weight of the animals that received propylgallate; III -- butyloxyanisol; IV -- butyloxytoluol.

((text continued)): The cholinesterase activity was reduced in animals in whose diet there was mixed in fat with BNT and PG.

By the end of the experiment, the activity was as follows: in control animals -- there was 19.7 - 25.4% disintegrated acetylcholinchloride; inthe group that received BNT, -- 10.4 - 16.3%; in the group that received PG, -- 13.2 - 16.3%; in the group that received BNA, there was no distinction from the control group. No deviations were noted in the morphological composition of the blood and in examining the urine of the experimental (test) animals. The prothrombin time of

all the animals in the experimental (test) groups did not differ from that of the control group.

((Two graphs on pg. 27 of text, in the right-hand column)) ((to the left of the upper graph)): % fat; ((underneath the same graph)): Fig. 2. Fat content in the liver (for a dry substance).

I -- control group; II -- animals that had received butyloxyanisol; III -- those that had received butyloxytoluol; IV -- those that had received propylgallate.

((to the left of the lower graph)): M2% lipoid phosphorus; ((underneath this graph)): Fig. 3. Content of lipoid phosphorus in the liver (for a dry substance). Legend (conventional symbols) the same as in Fig. 2. ((text continued)):

In dissecting the animals groups no pathological-anatomical changes were observed. The weight of the liver in rats that had received BNA and BNT was greater than that in the control ones and than that of the group that had received PG. In the histological examination of the internal organs (the cerebrum (brain), heart, lungs, stomach, duodenum, small intestine, caecum (blind gut), colon, liver, spleen, kidneys, urinary bladder, and the sex organs), all three experimental (test) groups did not indicate any significant changes in comparison with the control animals. Only in one rate (No. 15) from the propylgallate group was there an edema of the stroma in a testicle, with a discontinuance of spermatogenesis. In another rat (No. 17) of the same group, the villi of the small intestine were edematic with markedly dilated lymphatic capillaries. Pathomorphological investigations were carried out in the Nutritional Hygiene Section of the Nutrition Institute of the AMN SSSR Yu. P. Kukelum (Yu. P. Kukelem Academy of Medical Sciences of the U.S.S.R.).

The fat content in the liver was identical with that in the control group and with that in the groups that had received BNA and PG (from 14.6 to 20.6%). The liver of the rats, however, that had received BNT, contained an increased amount of fat (20.8 - 27.4%) (Fig. 2). At the same time, in the liver of these animals there was little lipoid phosphorus (Fig. 3). This points to the fact that, in animals that had received BNT, there was an impairment in the synthesis of phos-

pholipids and in the processes of breaking up and secretion of neutral fat from the liver (true, still without any histologically disclosed fatty infiltration of the liver). There was a somewhat reduced content of lipoid phosphorus also in those animals that had received BNA.

The percentage content of dry substances and glycogen was approximately the same in all four groups.

The acute and subacute experiments that had been carried out yield a basis for drawing the following conclusions.

1. Phenol antioxidants (butyloxyanisol, butyloxytoluol, and propylgallate) do not pertain to highly toxic substances(LD_{50} is greater than 1000 milligrams for 1 kilogramof body weight), and their toxicity is close with reference to one another.

2. Subacute experiments have shown that phenol antioxidants, even though they did not bring about any histological changes in the organs with the administration of large doses, represent far from neutral substances for the organism of animals:

a) experimental (test) rats lagged in weight in comparison with the control ones; this points to the fact that the doses of preparations that had been fed brought about an impairment in the processes of assimilation in the organism of the animals;

b) the antioxidants under study lowered the level of the oxidizing processes in the organism (a reduction in the activity of the oxidizing enzymes);

c) butyloxytoluol and propylgallate reduced the activity of cholinesterase in the blood serum;

d) Butyloxytoluol showed the greatest toxicity. Chemical examinations of the liver disclosed that butyloxytoluol manifests a harmful action on the liver, impairing the process of phospholipoid synthesis, and also, that entailed in the breaking up and secretion of neutral fat from the liver.

3. For a definitive conclusion with regard to the possibility of introducing phenol antioxidants into industrial practice, chronic experiments are being conducted, at the present time, with small doses of the preparations. This is necessary in order to eliminate completely the possible toxic action (effect) on an

individual of fat containing butyloxyanisol, butyloxytoluol and propylgallate.

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Figure 1

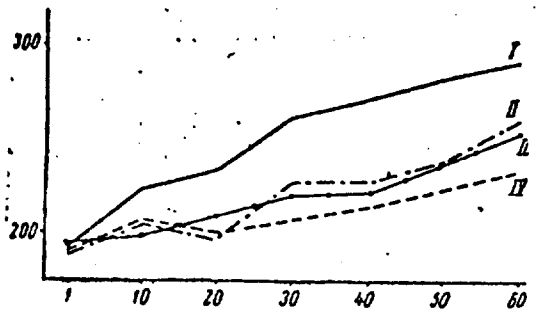


Figure 2

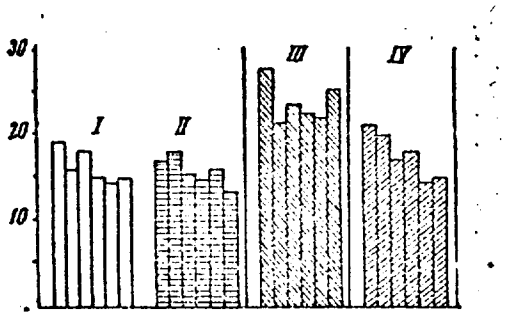
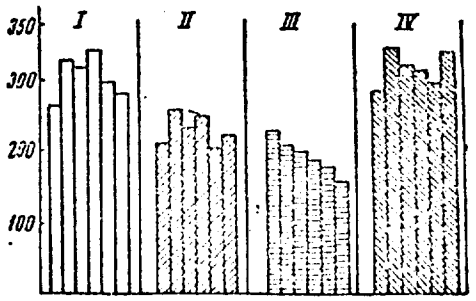


Figure 3



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ТОКСИКОЛОГИЧЕСКАЯ ХАРАКТЕРИСТИКА ФЕНОЛЬНЫХ АНТИОКИСЛИТЕЛЕЙ ПИЩЕВЫХ ЖИРОВ (ОСТРЫЕ И ПОДОСТРЫЕ ОПЫТЫ)

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Из кафедры гигиены питания (зав. — проф. Ф. Е. Будагян) Центрального института усовершенствования врачей, Москва

Жиры, являясь высокоценным продуктом питания, подвергаются быстрой порче, которая в основном обусловлена окислительными процессами. Окисление при низких температурах несколько замедляется, но не прекращается, поэтому даже в холодильнике жиры можно сохранять не дольше полугода. В процессе окисления жиры теряют свои вкусовые и питательные свойства и могут оказывать вредное действие (И. Смородинцев, 1947; Burr и Barues, 1943; Johnson, Perkins, Sugal и Kummerow, 1957, и др.).

Окончательно еще не выяснено, сами ли продукты окисления жира (перекиси, полимерные продукты) оказывают прямое вредное действие или при окислении жиров (при прогоркании) окисляются и инактивируются такие важные составные части рациона, как витамины A, D, E, K, пантотеновая кислота, пиридоксин, биотин, аскорбиновая кислота и жизненно необходимые ненасыщенные жирные кислоты (линолевая, линоленовая, арахидоновая). Патологические явления, развивающиеся при кормлении крыс прогоркшей экспериментальной диетой, возможно объясняются недостатком этих питательных веществ в рационе (Quackenbusch, 1945; Greenberg и Frazer, 1953; Witting, Hishida, Johnson и Kummerow, 1957).

Имеются также данные о том, что глубоко окисленные жиры обладают канцерогенным действием (Lavik и Baumann, 1941, 1943; Roffo, 1938). Спектр термически окисленного жира имеет сходство со спектром канцерогенных дериватов фенантрена (Roffo, 1938).

Одним из эффективных способов предохранения жиров от окисления является введение в жиры антиокислителей. Наибольший интерес проявляется к фенольным антиокислителям, как наиболее эффективным: бутилоксианизолу, бутилокситолуолу и эфирам галловой кислоты. В ряде стран эти антиокислители широко используются в пищевой промышленности (Tollenaar и Vos, 1956).

В настоящее время в Советском Союзе Всесоюзным научно-исследовательским институтом мясной промышленности и Всесоюзным научно-исследовательским институтом жиров предлагается вводить бутилоксианизол (BHA), бутилокситолуол (BHT) и пропилгаллат (PG) в топленые жиры и растительные масла. Антиокислители рекомендуется вводить в количестве 0,01—0,02%. При таком количестве бутилоксианизола или бутилокситолуола жиры повышают свою стойкость в 3—5 раз, а при добавлении галлатов — до 10 раз (Н. Эмануэль, Д. Кнорре, Ю. Лясковская и В. Пиульская, 1955, 1958). Органолептических изменений жира эти антиокислители в указанных количествах не вызывают.

Когда эти препараты были предложены для применения их в пищевой промышленности, встал вопрос об изучении их токсичности. Изучением токсичности бутилоксианизола занимались Lehmann, Fitzugh, Helson и Woodard (1951), Wilder, Ostby и Gregory (1956) и др. Takata, Nagata (1952), Deichmann, Clemmer, Rakoczy и Bianchine (1955) изучали бутилокситолуол. Токсичность галлатов определяли Boehm и Williams, (1943), Orten, Knyper и Smith (1948), Allen и De Eds (1951), Roy (1953), Sporn и Peretianu (1956). Работами вышеперечисленных авторов были установлены LD_{50} этих препаратов для мышей, крыс, кроликов, морских свинок, кошек, причем метод установления этой до-

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зы различен у каждого автора: одни устанавливали ее при подкожном введении, другие — при внутрибрюшинном, третьи — при энтеральном; вводились растворы различной концентрации. Понятно, что и полученные результаты отличаются друг от друга. При длительном введении (1—3 месяца) больших доз фенольных антиокислителей (около 1% от веса корма) авторами отмечалось нарушение роста и развития животных. Пропилгаллат при таких концентрациях приводил, кроме того, к уменьшению количества гемоглобина крови и к патологическим явлениям со стороны почек (появление в моче белка). Бутилоксианизол при концентрации 0,25 г на 1 кг веса у собак вызывал дегенеративные изменения паренхиматозной ткани печени. Скармливание высших галлатов (октил, додецил) самкам крыс в концентрации 0,5% от веса корма привело к нарушению лактации и оказывало вредное влияние на молодое поколение. Следует отметить, что все авторы в подострых опытах применяли очень ограниченное число тестов: вес животных, исследование крови и патоморфологические исследования органов. Только Deichmann и др. (1955), изучая токсичность бутилокситолуола, определяли протромбиновое время и измеряли кровяное давление. Нам кажется, что количество использованных авторами тестов недостаточно, чтобы судить о функциональном состоянии организма. В своих опытах мы пытались возможно шире исследовать различные стороны жизнедеятельности организма.

Для сравнительной токсикологической характеристики изучаемых препаратов (бутилоксианизол, бутилокситолуол, пропилгаллат) были поставлены острые опыты на крысах и мышах. В опыт были взяты растущие здоровые животные (самцы и самки). Антиокислители вводились утром, натощак, в растворе подсолнечного масла непосредственно в пищевод из шприца через металлический зонд, представляющий собой обыкновенную толстую инъекционную иглу с напаянной оливой на конце. Животные, оставшиеся в живых после введения препарата, находились под наблюдением в течение 20 дней. Животным вводили различные количества изучаемых препаратов (7—8 доз). Каждую дозу получало по 8 крыс и 10 мышей.

Проведя затравку животных различными дозами изучаемых препаратов, мы установили токсичность этих веществ от минимально токсической дозы до абсолютно смертельной. Затем, проведя принятое в

Таблица 1

Результаты определения токсических и смертельных доз изучаемых антиокислителей

Антиокислители	Крысы			Мыши	
	минимально токсическая доза в мг на 1 кг веса	LD_{50} мг на 1 кг веса	LD_{100} мг на 1 кг веса	LD_{50} мг на 1 кг веса	LD_{100} мг на 1 кг веса
Бутилоксианизол (BHA)	1 000	2 900	4 500	1 250	3 000
Бутилокситолуол (BHT)	500	2 450	3 500	2 000	2 500
Пропилгаллат (PG) . . .	500	2 600	3 500	1 700	2 500

токсикологии интегрирование по Беренсу (Н. С. Правдин, 1947; В. С. Шадурская, 1953), построили кривую смертности и по ней определили LD_{50} всех трех изучаемых антиокислителей для крыс и мышей. Результаты опытов представлены в табл. 1.

Из полученных данных видно, что токсичность этих препаратов очень близка друг к другу и что фенольные антиокислители сравнительно мало токсичны.

Подострые опыты были поставлены с целью выявления функциональных нарушений под влиянием изучаемых препаратов, в заведомо больших, аггравированных дозах (сравнительно с теми, которые могут

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попасть в организм человека). В случае обнаружения таких отклонений фиксировали внимание именно на этих тестах в хронических опытах при окончательном решении вопроса о возможности введения фенольных антиокислителей в пищевые жиры.

В подострых опытах использовались четыре группы крыс: первая группа получала в рационе жир с ВНА в дозе $^1/_5$ от LD_{50}, т. е. 600 мг на 1 кг веса тела; вторая группа получала ВНТ, первоначально $^1/_5$ от LD_{50}, т. е. 500 мг на 1 кг веса; в дальнейшем эта доза была уменьшена до 250 мг на 1 кг веса тела из-за плохой поедаемости корма при более высоком содержании ВНТ; третья группа получала $^1/_5$ от LD_{50} PG — 500 мг на 1 кг веса тела; четвертая группа была контрольной.

Антиокислители вводились в свиной топленый жир, который примешивался к основному корму. Контрольная группа получала чистый лярд. Свиной топленый жир и препараты антиокислителей, необходимые для постановки опытов, были предоставлены нам Всесоюзным научно-исследовательским институтом мясной промышленности.

Каждая крыса содержалась в отдельной клетке, что позволяло следить за количеством съеденного корма и жира с антиокислителями.

Для наблюдения за функциональным состоянием подопытных животных мы остановились на следующих показателях.

1. Ежедневное наблюдение за животными.
2. Взвешивание животных один раз в 10 дней.
3. Проведение морфологического исследования крови, поскольку известно, что фенол и его производные часто вызывают патологию кровотворения. При морфологическом исследовании производился подсчет форменных элементов: эритроцитов, ретикулоцитов, тромбоцитов, лейкоцитов; определялось содержание гемоглобина и лейкоцитарная формула.
4. Исходя из того, что окислительные процессы лежат в основе большинства биохимических реакций организма и что антиокислители, возможно, и в живом организме оказывают влияние на процессы окисления, исследовалась активность окислительных ферментов, а именно каталазы и пероксидазы. Эти ферменты были выбраны еще и потому, что Tappel и Marr (1954), изучая in vitro действие пропилгаллата и нордигидрогвайаретовой кислоты (тоже фенольного антиокислителя) на 14 энзиматических систем, нашли, что они в концентрации 1×10^{-4} M тормозят деятельность каталазы и пероксидазы. Тормозящее действие фенольных соединений на каталазу показали также Goldacre и Galston (1953). Каталаза определялась по методу Баха и Зубковой и выражалась в виде показателя каталазы. Пероксидаза определялась по окислению индигокармина и выражалась во времени (секундах), необходимом для его окисления.
5. Исследовалась холинэстеразная активность сыворотки крови, которая, по мнению физиологов (К. М. Быков, 1949; Х. С. Коштоянц, 1946), тесно связана с функциональным состоянием нервной системы.
6. Исследовалось протромбиновое время (зависит от количества протромбина, синтезируемого в печени). Клиницисты признают ценность этого определения для выявления функциональных нарушений печени.

Все выбранные показатели проверялись у животных до опыта, а после дачи антиокислителей — каждые 2 недели. Животные находились на опыте от 68 до 82 дней. В конце опытного периода все животные забивались обезглавливанием. Производилось патологоанатомическое вскрытие и гистологическое исследование внутренних органов. Помимо патогистологического исследования, печень подвергалась химическому изучению: определялось содержание сухих веществ, количество гликогена, жира и липоидного фосфора для выявления возможного нарушения некоторых сторон углеводного и жирового обмена в этом органе.

За весь опытный период каждое подопытное животное получило следующее количество препаратов: ВНА — от 5 до 7 г, ВНТ — от 2 до 4 г, PG — от 4 до 6 г.

Изменений в поведении и внешнем виде подопытных животных отмечено не было. Взвешивание животных показало, что до дачи антиокислителей животные всех групп одинаково хорошо прибавляли в весе. С началом же введения антиокислителей в рацион вес подопытных животных начал значительно отставать от веса контрольных (рис. 1). Отставание в весе животных подопытных групп особенно было выражено в первый месяц опыта и меньше — во второй месяц.

У животных, получавших в рационе ВНА и ВНТ, было выявлено снижение активности каталазы крови. У контрольных крыс к концу опытного периода активность каталазы составляла 9,5—13,5 мг перекиси водорода, в группе ВНА — 6,7—8,8 мг, в группе ВНТ к 43-му дню опыта — 6,1—8,8 мг; к 60-му дню активность несколько повысилась, но не достигла уровня каталазы контрольных животных. Пропилгаллат не вызвал изменений активности каталазы.

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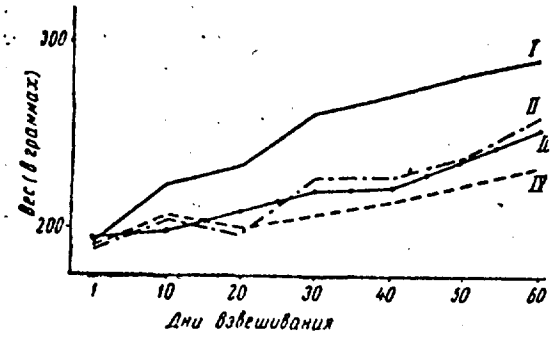

Рис. 1. Изменение веса крыс в течение опытного периода.

I — вес контрольных животных; *II* — вес животных, получавших пропилгаллат; *III* — бутилоксианизол; IV — бутилокситолуол.

Понизилась активность холинэстеразы у животных, в рацион которых примешивался жир с BHT и PG.

К концу опыта активность была следующей: у контрольных животных — 19,7—25,4% разрушенного ацетилхолинхлорида; в группе, получавшей BHT, — 10,4—16,3%; в группе, получавшей PG, — 13,2—16,3%; в группе, получавшей BHA, не отличалась от контрольной. Каких-либо отклонений в морфологическом составе крови и при исследовании мочи подопытных животных отмечено не было. Протромбиновое время всех животных подопытных групп не отличалось от такового контрольной группы.

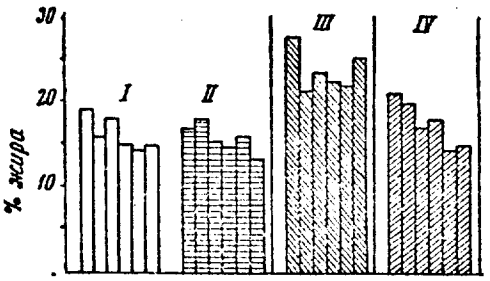

Рис. 2. Содержание жира в печени (на сухое вещество).

I — контрольная группа; *II* — животные, получавшие бутилоксианизол; *III* — получавшие бутилокситолуол; *IV* — получавшие пропилгаллат.

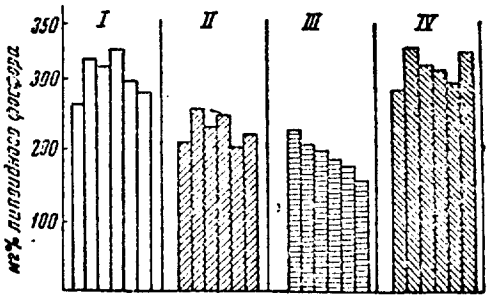

Рис. 3. Содержание липоидного фосфора в печени (на сухое вещество). Условные обозначение те же, что и на рис. 2.

При вскрытии животных грубых патологоанатомических изменений обнаружено не было. Вес печени крыс, получавших BHA и BHT, был выше, чем контрольных, и группы, получавшей PG. При гистологическом исследовании внутренних органов (головного мозга, сердца, легких, желудка, двенадцатиперстной кишки, тонкого кишечника, слепой и ободочной кишки, печени, селезенки, почек, мочевого пузыря, половых органов) все три подопытные группы не дали сколько-нибудь значительных изменений сравнительно с контрольными животными. Только у одной крысы (№ 15) из группы пропилгаллата имелся отек стромы семенника с прекращением сперматогенеза. У другой крысы (№ 17) этой же группы ворсинки тонкого кишечника были отечны с резко расширенными лимфатическими капиллярами. Патоморфологические исследования были проведены в Отделе пищевой гигиены Института питания АМН СССР Ю. П. Кукелем.

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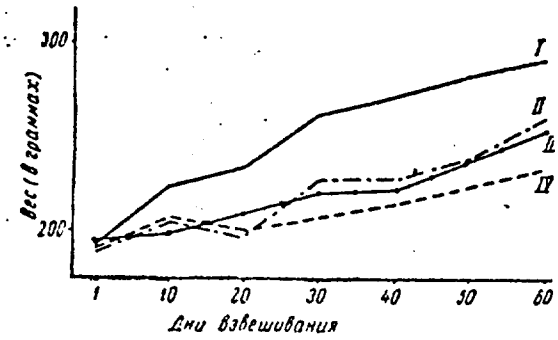

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Понизилась активность холинэстеразы у животных, в рацион которых примешивался жир с ВНТ и PG.

К концу опыта активность была следующей: у контрольных животных — 19,7—25,4% разрушенного ацетилхолинхлорида; в группе, получавшей ВНТ, — 10,4—16,3%; в группе, получавшей PG, — 13,2—16,3%; в группе, получавшей ВНА, не отличалась от контрольной. Каких-либо отклонений в морфологическом составе крови и при исследовании мочи подопытных животных отмечено не было. Протромбиновое время всех животных подопытных групп не отличалось от такового контрольной группы.

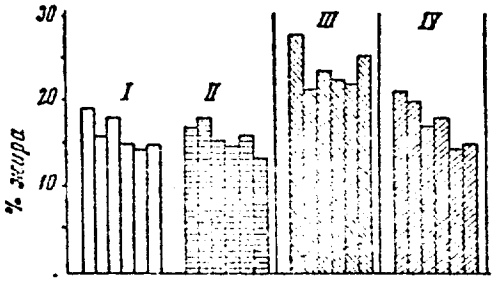

Рис. 2. Содержание жира в печени (на сухое вещество).

I — контрольная группа; *II* — животные, получавшие бутилоксианизол; *III* — получавшие бутилокситолуол; *IV* — получавшие пропилгаллат.

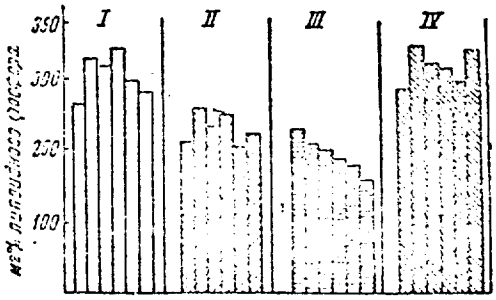

Рис. 3. Содержание липоидного фосфора в печени (на сухое вещество). Условные обозначение те же, что и на рис. 2.

При вскрытии животных грубых патологоанатомических изменений обнаружено не было. Вес печени крыс, получавших ВНА и ВНТ, был выше, чем контрольных, и группы, получавшей PG. При гистологическом исследовании внутренних органов (головного мозга, сердца, легких, желудка, двенадцатиперстной кишки, тонкого кишечника, слепой и ободочной кишки, печени, селезенки, почек, мочевого пузыря, половых органов) все три подопытные группы не дали сколько-нибудь значительных изменений сравнительно с контрольными животными. Только у одной крысы (№ 15) из группы пропилгаллата имелся отек стромы семенника с прекращением сперматогенеза. У другой крысы (№ 17) этой же группы ворсинки тонкого кишечника были отечны с резко расширенными лимфатическими капиллярами. Патоморфологические исследования были проведены в Отделе пищевой гигиены Института питания АМН СССР Ю. П. Кукелем.

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Содержание жира в печени было одинаковым в контрольной группе и в группах, получавших BHA и PG (от 14,6 до 20,6%). Печень же крыс, получавших BHT, содержала увеличенное количество жира (20,8—27,4%) (рис. 2). В то же время в печени этих животных было мало липоидного фосфора (рис. 3). Это указывает на то, что у животных, которые получали BHT, нарушены синтез фосфолипидов, процессы расщепления и вывода нейтрального жира из печени (правда, еще без гистологически выявляемой жировой инфильтрации печени). Несколько снижено содержание липоидного фосфора и у животных, получавших BHA.

Процент содержания сухих веществ и гликогена был приблизительно одинаков во всех четырех группах.

Проведенные острые и подострые опыты дают основание сделать следующие выводы.

1. Фенольные антиокислители (бутилоксианизол, бутилокситолуол, пропилгаллат) не относятся к сильнотоксичным веществам (LD_{50} больше 1000 мг на 1 кг веса тела), и токсичность их близка друг к другу.

2. Подострые опыты показали, что фенольные антиокислители, хотя и не вызвали гистологических изменений в органах при даче больших доз, являются далеко не безразличными веществами для организма животных:

а) подопытные крысы отставали в весе от контрольных; это указывает на то, что скармливаемые дозы препаратов вели к нарушению процессов ассимиляции в организме животных;

б) изучаемые антиокислители понижали уровень окислительных процессов в организме (понижение активности окислительных ферментов);

в) бутилокситолуол и пропилгаллат понижали активность холинэстеразы сыворотки крови;

г) наибольшую токсичность показал бутилокситолуол. Химические исследования печени выявили, что бутилокситолуол оказывает вредное воздействие на печень, нарушая процесс синтеза фосфолипидов, расщепления и вывода нейтрального жира из печени.

3. Для окончательного вывода о возможности введения фенольных антиокислителей в производственную практику в настоящее время ведутся хронические опыты с небольшими дозами препаратов. Это необходимо для того, чтобы полностью исключить возможное токсическое действие на человека жира, содержащего бутилоксианизол, бутилокситолуол и пропилгаллат.

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A TOXICOLOGIC CHARACTERISTICS OF PNEHOLIC ANTIOXIDANTS OF ALIMENTARY FATS

I. A. Karplyuk (Moscow)

Summary

In experiments stages for the study of acute toxicity phenolic antioxidants (butil-oxianisol, butyloxitoluol and propalgallate) minimal toxic doses of LD 50 and LD 100 have been determined for two kinds of animals viz.—mice and rats.

In subacute experiments lasting for 2.5 months with the preparation dosed in the amounts of 1/5—1/10 from LD 50—a delayed increase of the weight has been registered in all experimental animals, accompanied by a reduced ferment activity, viz.—catalases, perioxidases and cholinesterases. The butaloxitoluol produced disturbed fat metabolism in the liver.

Chronic experiments are being conducted at present to arrive at the final conclusion as to the possibility of introducing phenol antioxidants into the industrial practice.

ГИГИЕНИЧЕСКИЙ АНАЛИЗ ПИТАНИЯ УЧАСТНИКОВ ВСЕСОЮЗНЫХ СПАРТАКИАД УЧАЩИХСЯ ШКОЛ

Г. М. Краковяк

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В интересах укрепления здоровья и более широкого охвата детей спортом ежегодно (с 1954 г.) проводятся всесоюзные спартакиады учащихся школ.

В предшествовавших спартакиадам соревнованиях каждый год принимало участие свыше 10 миллионов школьников.

Питание юных спортсменов изучалось нами на I (1954 г., Ленинград), II (1955 г., Киев) и IV (1957 г., Рига) спартакиадах у 12 спортивных делегаций (1803 человека). Производилась гигиеническая оценка организации и режима питания, качественного и количественного состава пищи.

Полноценное питание имеет большое значение для роста и развития организма, выработки иммунитета, укрепления здоровья, повышения работоспособности.

У молодежи, занимающейся спортом, по сравнению со взрослыми обмен веществ относительно выше, поэтому затраченная энергия должна полностью восстанавливаться питанием (Г. М. Краковяк, 1956). Пища юного спортсмена должна быть более калорийной (75—80 калорий на 1 кг веса), чем у учащихся школ того же возраста (60—65 калорий на 1 кг веса), не занимающихся спортом. Она должна содержать 3—3,5 г белка на 1 кг веса при соотношении белков, жиров и углеводов 1 : 2 : 4, или 14% белков, 30% жиров и 56% углеводов суточной калорийности.

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The Metabolism of Phenolic Antioxidants (*)

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(For Summary in English see original publication)

Let us begin by considering to what extent biochemical investigations of metabolic transformations can contribute to the clarification of the question whether or not a certain chemical substance is suitable as additive for foods or feeds.

Most animal cells contain systems whose function seems to be to protect the animal from toxic substances which may be taken up into the body with the food, by contact, respiration, etc. As a rule, it may be considered that non-ionic, liposoluble substances can most easily penetrate through the cell membrane and that these substances may cause organic or functional damage in the cell. An important activity of the detoxication mechanisms situated in the cell itself therefore is to modify extraneous chemical substances in such a way that they are given an ionic charge and become water-soluble. These derivatives can then be excreted through the kidney with the urine or secondarily via the liver and bile duct with the feces. These biologic transformations are brought about by enzyme groups, found mainly in the microsomes.

Although the term metabolism should strictly be applied only to designate these intracellular modifications of the molecular structure, it is sometimes extended to resorption fron the intestine, storage in the tissue, and the kinetics of excretion. The information obtained from studies of these processes is of great value for the toxicological exploration of food additives. For if it can be proven that in quantities as normally absorbed with the food the respective substances are not resorbed from the digestive tract at all, or if that is the case, are metabolized in a very short time and excreted without storage in any organ, these are weighty arguments which might prevail

on the State authorities ruling on the permissibility of these substances to ease up on some of their stricter requirements in view of the data obtained from animal tests. Especially when it can be demonstrated that no substances of known or presumed carcinogenic effect are released into the tissue by the processes of metabolism, applications for approval of the respective substance as food additives are more likely to find favor with the authorities.

The value of such investigations on metabolism lies further in the fact that the results of toxicological animal tests can be applied to man with greater certainty. As a rule, differences in the sensitivity of different animal species to the toxicity of a chemical substance are attributable less to a different sensitivity of the receptor organ than to inherent differences of the metabolic path. The maximum permissible daily dose for man is determined on the basis of experimentally obtained "tolerance values" for one or more animal species, i.e. the doses at which even after prolonged administration no action is observable. It is customary in this connection to apply a safety factor of at least 100. It is taken so high partly for the reason that the specific human sensitivity to the examined substance cannot be determined with certainty. Now if approximately the same quantitative and qualitative values and paths can be demonstrated in human metabolism as in the animal species used in the experiment, then this safety factor can perhaps be reduced.

The intracellular enzymes which cause the metabolism have a remarkable ability of "neutralize" a great variety of extraneous substances. While there is as yet no direct proof that the transformation of phenolic antioxidants in the organism is related to their ability to stop free-radical chain reactions, such an effect in the organism can be inferred from certain animal experiments: In animals from whose feed the natural antioxidant tocopherol (vitamin E) had been excluded, they successfully prevented the autoxidation of tissue lipoids.

How then does the transformation of the substituted phenols into water-soluble substances and their anionic charge take place? Usually, unless this process is inhibited spatially, the phenol group is coupled with glucuronic

acid to aryl glucosiduronates or with sulfuric acid to monoester sulfates. If there is no reacting hydroxyl group, the ring can be hydroxylized by microsome oxidase, just as is the case in the transformation of benzene to phenol. In the presence of certain other ring substituents, a methoxy group can be demethylized to the respective phenol. Alkyl groups can be oxidized to carboxyl groups. Thus benzoic acid is formed from toluene. The acid is then coupled either with glucuronic acid to an aryl glucosiduronate (ester glucuronide) or with glycin to a hippuric acid derivative. On the other hand, the metabolic enzymes can cause changes which inhibit solubilization and elimination from the organism; phenolic hydroxyl groups can be methylized or carboxyl groups destroyed by elimination of carbon dioxide. Where the conversion in the metabolism can proceed in more than one way, the quantity ratio of the end products depends on reaction-kinetic factors which may be different in different animal species, but also in animals of the same species according to sex, age, nutrition and perhaps also "stress".

Until about ten years ago, in experimental studies of the metabolism of new compounds, large single doses or several smaller doses were given to the test animals, usually rats, rabbits or dogs. Then the urine was collected and fractionated by suitable methods to be able to isolate the metabolic products in a pure state and to characterize them as to melting point, elementary analysis, etc. In such studies it was usually possible only to identify the most important metabolic products, and if it was possible to recover more than 50% of the originally administered substance in the form of derivatives from the urine, this was already an extraordinary success. In recent times this work has been greatly facilitated by the introduction of radioactive indicators. The substance to be tested is synthetized at a suitable point of the molecule with a weak beta-ray emitter, usually C^{14} or tritium, and then administered in relatively small doses, which more or less correspond to the quantities present in the food. The radio-chemical methods are so sensitive that one can operate with radioactive doses which are entirely harmelss to animals - and also to man. The first step is the examination of the radioactivity curves of the

excrements and possibly also of the exhaled air and the establishment of a "balance" of the quantity administered, excreted, and stored in the tissue. Also the elimination through the bile into the intestine can be examined by introducing a cannula into the bile duct.

A good illustration of the value of radio-chemical methods is offered by the studies recently carried out in our laboratories on the metabolism of 2,6-di-tert.-butyl-4-methylphenyl (butylated hydroxytoluene, BHT.) In earlier work, only a relatively small portion of an orally administered dose could be detected in the urine, and no trace at all in the feces. Tissue storage was inferred, an assumption which created serious doubts about the fitness of this substance as food additive. With the use of chemical methods we showed, however, that rats which had been fed a BHT-containing diet contained little BHT in the body fat and in the liver, and that also these slight quantities disappeared from these organs after a short time when the rats were put on a normal diet again. We then gave the rats a dose of BHT which had been tagged with C^{14} at the tertiary butyl groups. In this way we were able to recover within four days up to 90% from the excretions, in large part from the feces. An examination of the tissues, carried out 2 and (illegible) days, respectively, after administration, showed only weak radioactivity, and the time during which half of the BHT had been excreted from the organism was estimated at less than 2 days. The highest radioactivity occurred in the intestine. Since, as we had shown before, the given dose is excreted for the most part through the bile, the explanation seems to be that the excretion of the BHT is delayed somewhat in the internal circulation between liver and intestine, in that the BHT derivatives excreted with the bile into the intestine are partly resorbed and returned to the liver, whence they pass into the intestine a second time. Experiments in which C^{14}-tagged BHT was given to man did not, however, indicate such an enterohepatic cycle. More than 50% of the dose were found in the urine within 24 hours; on the second day the excretion diminished perceptibly and was then present in ever smaller amount for several weeks. A similar antioxidant, 3-tert.-butyl-4-hydroxyanisole (butylated hydroxyanisole, BHA) is ex-

creted in the urine of the animal and of man for the most part within 24 hours. In man this first excretion is then followed by a long-lasting small excretion in the urine, as we observed it with BHT. It may be that in both antioxidants this delayed excretion represents the release of small quantities of metabolites which had been stored in the tissues.

Also for the qualitative examination of metabolic processes radioactive isotopes are extensively used today. The urine or also other biological substances are fractionated by various methods, usually by organic solvents (liquid extraction), ion exchange resins and column chromatography, and the presence of metabolic products is determined in each phase by evaluations of the radioactivity in the different fractions. For further isolation and separation of the metabolic products thin layer chromatography is preferred today, the radioactive points being localized by autoradiography. The individual metablic products can be determined by infrared spectroscopy or mass spectrometry or also by measurements of the nuclear magnetic resonance. To confirm the result of the determination, a substance of the same hypothetical structure is synthetized by a known method and compared with the product of natural metabolism using suitable chemo-phsical methods.

On the one hand, the use of radioactive indicators has considerably facilitated the determination of the main products of the metabolism. But on the other hand, it brings up new problems because we now find with their aid many other products which occur in smaller amounts and were formerly easily overlooked. In the oxidation of a methyl group to a carboxyl group, for example, small amounts of the respective intermediate alcohol and aldehyde and their derivatives are sometimes separated.

In Germany, tocopherol (vitamin E) is at present the only antioxidant which, alone or in combination with synergists, may be added to edible fats. In other countries one is more generous with the use of antioxidants because it has been realized that when fats become rancid, not only their taste is impaired, but also they may be injurious to health if taken in larger amount. In 1962, a joint committee of the FAO and of the World Health Organization

(WHO) published a list of the maximum permissible daily doses for antioxidants, to give the various countries guidelines for the establishment of limit values in the use of these substances as food additives. These values (see table below) are either "absolute", i.e. there is sufficient toxicological evidence that the stated quantities may be released everywhere without hesitation, or "conditional". In the latter case, a group of experts should decide on their approval in each country taking into consideration the local diet and other (e.g. climatic) factors.

Permissible daily doses of antioxidants in man

	Daily dose per body weight (mg/kg)	
Phenolic antioxidant	Absolute	Conditional
Butylated hydroxyanisole (BHA)	0-0.5	0.5-2
Butylated hydroxytoluene (BHT)	0-0.5	0.5-2
Propyl gallate	0-0.2	0.2-0.5
Octyl gallate	0-0.2	0.2-0.5
Dodecyl gallate	0-0.2	0.20.5
Tocpherol (Vitamin E)	0-1	1 - 2

Excerpt from the technical report of the World Health Organization No. 228, 1962, amended 1965

Let us try to summarize in a few words our present knowledge of the behavior of the most important phenolic antioxidants in the metabolism. The behavior of BHA has been examined on different animal species and also on man. In the rat and in the rabbit it is readily resorbed and most of it is excreted again after a short time with the urine, namely coupled directly with glucuronic acid, in lesser degree with sulfuric acid. In rats moreover a small portion is demethylated, so that formation of the respective phenol occurs. The conversion in human metabolism occurs similarly as in the rat and rabbit, except that so far no demethylation has been observed. In the dog, however, the processing in the metabolism differs essentially from the mode just described. Apparently, [illegible] half is resorbed from the intestine, and the greater part thereof is

excreted after coupling with sulfuric acid. Besides, a considerable demethylation and hydroxylation of the molecule was observed. From these results the conclusion was drawn that the dog is less suitable as test animal for toxicological studies to determine the BHA tolerance values to be established for man than the rat or the rabbit.

Also BHT is easily resorbed from the intestine, but apparently no phenolic hydroxyl group is available in this substance for coupling, probably because of the adjacent tertiary butyl groups. In the rabbit, the cyclic methyl group is oxidized to carboxylic acid, which is then excreted after coupling with glucuronic acid and glycin. There is excreted also the glucuronic acid derivative of an alcohol which probably is formed by oxidation of a tertiary butyl group. In the rat we have shown that little alcohol is excreted and that the main reaction path leads to a carboxylic acid by oxidation of the cyclic methyl group. The principal metabolite in man is the glucuronide of an oxidation product of BHT and is not yet identified in detail. To this is added, as in the rat, a relatively small quantity of a carboxylic acid, and it is probable that the enterohepatic cycle, which we have observed in the rat, depends on the presence of this acid.

Of the three gallic acid esters, propyl gallate has been examined in greatest detail. This compound is hydrolyzed to free gallic acid, which then methylates at the 4-hydroxyl group and - coupled with glucuronic acid - yields the principal metabolic product in the urine.

Tocopherol or vitamin E is a substituted phenol of very complex construction. The mechanism of the resorption of this substance from the digestive tract is quite unusual, probably because an essential constituent of food is involved. It takes long before a single dose is excreted; in the end the substance appears in the urine, but in a form which no longer shows any vitamin E activity. As far as I know, it has not yet been clarified how the transformation of this substance takes place in the organism.

Antioxidants are also widely used to prevent autoxidation of fats in feeds.

vention of contamination of the products intended for human consumption (meat, eggs, dairy products). The most important phenolic antioxidants used for this purpose are BHT and BHA. Pertinent examinations have shown that such a treatment of the feed in no way endangers human health.

Phenolic antioxidants have still another application: They serve to improve the durability of polyolefin plastics. Now when such plastics are used for the packaging of foods, the possibility exists that the antioxidant passes from the package into the food during storage. However, usually antioxidants of high molecular weight are used for packaging purposes, not only in order to reduce this kind of migration, but also so that the antioxidant will not volatilize during thermal treatment of the plastic. Several of these additions have been tested in animal experiments, and it was found that only very small amounts are resorbed from the intestine.

To summarize, it is seen that the examination of the behavior of chemical substances in human and animal metabolism offers interesting insights into the mechanisms with which the organism "neutralizes" extraneous substances absorbed with the food. These biochemical investigations can also give valuable information on which substances may be used as food additives without endangering human health.

(*) Read on the occasion of the DGF Symposium in Münster (Westf.) on October 13, 1965.

Translated by Carl Demrick Associates, Inc./LH/db

Der Metabolismus phenolischer Antioxydantien*

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Der Stoffwechsel von C^{14}-markiertem butyliertem Hydroxytoluol und butyliertem Hydroxyanisol wurde radiochemisch untersucht. Beide Stoffe werden bei Ratte, Kaninchen und Mensch leicht vom Darm resorbiert, jedoch in kurzer Zeit zum größten Teil wieder ausgeschieden, wobei vor allem Glucuronsäurederivate der genannten Verbindungen bzw. ihrer Oxydationsprodukte im Harn und Kot auftreten. Dagegen wird beim Hund butyliertes Hydroxyanisol nur in geringem Maße vom Darm resorbiert und der resorbierte Teil hauptsächlich nach Kuppelung an Schwefelsäure ausgeschieden.

The Metabolism of Phenolic Antioxidants

The metabolism of C^{14}-labelled butylated hydroxytoluene and butylated hydroxyanisole was investigated radiochemically. Both substances are easily resorbed in the intestines of rats, rabbits and human beings but most of it is excreted again within a short time in urine and stools, especially as glucuronic acid derivatives of the said compounds and their oxidation products. In the case of dogs, however, butylated hydroxyanisole is resorbed in the intestine only to a small extent and the resorbed part is excreted mainly after being coupled with sulfuric acid.

Le métabolisme des antioxygènes phénoliques

On a étudié radiochimiquement, le métabolisme du butylhydroxytoluol et du butylhydroxyanisol marqués au C^{14}. L'une et l'autre de ces substances, aisément résorbées par l'intestin du rat, du lapin et de l'homme, sont cependant éliminées peu après dans l'urine et les fecés, à l'état de dérivés gluconuriques. Par contre, le taux de résorption du butylhydroxyanisol par l'intestin du chien est minime, la partie résorbée étant éliminée principalement après combinaison à l'acide sulfurique.

Метаболизм фенольных антиоксидантов.

Обмен бутилированного гидрокситолуола и бутилированного гидроксианизола, меченных с помощью ^{14}C, исследовался радиохимически. Оба вещества легко всасывались кишечником крысы, кролика и человека, после короткого времени же выделялись в большом количестве, причем в моче и кале прежде всего содержатся дериваты глюкуроновой кислоты или продукты окисления этих веществ. У собаки бутилированный гидроксианизол всасывается лишь незначительно и преимущественно выделяется после соединения с серной кислотой.

Zunächst seien einige Betrachtungen über die Frage vorausgeschickt, inwieweit biochemische Untersuchungen metabolischer Umwandlungen zur Klärung der Frage beitragen können, ob sich ein bestimmter chemischer Stoff als Zusatz für Lebens- oder Futtermittel eignet oder nicht.

Die meisten tierischen Zellen enthalten Systeme, deren Funktion anscheinend darin besteht, das Tier vor toxischen Stoffen zu schützen, die mit der Nahrung, durch Kontakt, Einatmen usw. in den Organismus aufgenommen werden können. In der Regel kann man davon ausgehen, daß nichtionische, fettlösliche Stoffe am leichtesten durch die Zellmembran eindringen und daß diese Stoffe unter Umständen organische oder funktionelle Schäden in der Zelle verursachen können. Eine wichtige Tätigkeit der in der Zelle selbst gelegenen Entgiftungsmechanismen besteht daher darin, chemische Fremdstoffe so zu modifizieren, daß sie eine ionische Ladung erhalten und wasserlöslich werden. Diese Derivate können dann durch die Niere mit dem Urin oder in zweiter Linie über die Leber und den Gallengang mit dem Kot ausgeschieden werden. Diese biologischen Umwandlungen werden durch Enzymgruppen bewirkt, die hauptsächlich in den Mikrosomen anzutreffen sind.

Obgleich die Ausdrücke Stoffwechsel oder Metabolismus streng genommen nur zur Bezeichnung dieser intrazellulären Abänderungen der Molekülstruktur angewandt werden sollten, werden sie mitunter auch auf die Resorption aus dem Darm, die Speicherung im Gewebe und die Exkretionskinetik ausgedehnt. Die Informationen, die aus Untersuchungen dieser Vorgänge gewonnen werden, sind für die toxikologische Erforschung von Lebensmittel-Zusätzen von großem Wert. Läßt sich nämlich der Nachweis erbringen, daß bei Mengen, wie sie üblicherweise mit der Nahrung aufgenommen werden, die betreffenden Stoffe überhaupt nicht aus dem Verdauungstrakt resorbiert werden, bzw. selbst wenn dies geschieht, in kürzester Zeit metabolisiert und ausgeschieden werden, ohne daß es in irgendeinem Organ zu einer Speicherung kommt, so sind dies gewichtige Argumente, die die staatlichen Stellen, die über die Zulässigkeit dieser Stoffe zu befinden haben, vielleicht dazu bewegen können, einige ihrer strengeren Anforderungen hinsichtlich der aus Tierversuchen gewonnenen Daten etwas zu lockern. Vor allem dann, wenn der Nachweis gelingt, daß durch die Vorgänge im Stoffwechsel keinerlei Substanzen mit bekannter oder mutmaßlicher karzinogener Wirkung ins Gewebe freigesetzt werden, finden Gesuche um Zulassung des betreffenden Stoffes als Lebensmittelzusatz bei amtlichen Stellen eher Gehör.

Der Wert solcher Untersuchungen über den Stoffwechsel liegt ferner darin, daß die Ergebnisse toxikologischer Tierversuche mit größerer Sicherheit auf den Menschen angewandt werden können. In der Regel sind Unterschiede in der Empfindlichkeit verschiedener Tierarten gegen die Toxizität eines chemischen Stoffes weniger auf eine unterschiedliche Empfindlichkeit des Rezeptororgans als vielmehr auf artspezifische Unterschiede des metabolischen Weges zurückzuführen. Die höchstzulässige Tagesdosis für den Menschen bestimmt man auf Grund von experimentell gewonnenen „Toleranzwerten" für eine oder mehrere Tierarten, d. h. der Dosen, bei denen auch nach langer Verabreichung keine Wirkung festzustellen ist. Hierbei ist es üblich einen Sicherheitsfaktor von wenigstens 100 einzusetzen. Dieser wird zum Teil deswegen so hoch angesetzt, weil sich die spezifische Empfindlichkeit des Menschen auf den untersuchten Stoff nicht mit Bestimmtheit feststellen läßt. Lassen sich nun beim Stoffwechsel des Menschen quantitativ und qualitativ etwa die gleichen Werte und Wege aufweisen wie bei der im Versuch verwendeten Tierart, dann kann dieser Sicherheitsfaktor vielleicht verringert werden.

Die intrazellulären Enzyme, die den Stoffwechsel bewirken, haben eine bemerkenswerte Fähigkeit, Fremdstoffe der verschiedensten Art zu „neutralisieren". Es liegen zwar noch keine direkten Beweise vor, daß die Umwandlung phenolischer Antioxydantien im Organismus mit ihrer Fähigkeit in Beziehung steht, Freiradikal-Kettenreaktionen zum Stillstand zu bringen; eine derartige Wirkung im Organismus läßt sich aber aus gewissen Tierversuchen erschließen: Bei Tieren, aus deren Nahrung das natürliche Antioxydans Tocopherol (Vitamin E) ausgeschaltet wurde, konnten sie mit Erfolg die Autoxydation von Gewebelipoiden verhindern.

Wie geht nun die Umwandlung der substituierten Phenole zu wasserlöslichen Stoffen und deren anionische Ladung vor sich? Meist wird die Phenolgruppe, sofern dieser Vorgang nicht

* Vortrag anläßlich der DGF-Vortragstagung in Münster (Westf.) am 13. Oktober 1965.

anderen Ländern ist man mit der Verwendung von Antioxydantien großzügiger, weil man zur Einsicht gekommen ist, daß bei Fetten, wenn sie ranzig werden, nicht nur der Geschmack beeinträchtigt wird, sondern bei Einnahme größerer Mengen auch die Gesundheit gefährdet werden kann. 1962 veröffentlichte ein gemeinsamer Ausschuß der FAO und der Weltgesundheitsorganisation (WGO) ein Verzeichnis der höchstzulässigen Tagesdosen für Antioxydantien, um den einzelnen Staaten Richtlinien für die Festsetzung von Grenzwerten bei der Verwendung dieser Stoffe als Lebensmittelzusätze an die Hand zu geben. Diese Werte (siehe nachstehende Tabelle) sind entweder „absolut", d. h. es liegt genügend toxikologisches Beweismaterial vor, daß die angegebenen Mengen überall ohne Bedenken freigegeben werden können, oder „bedingt". Im letzteren Fall soll in jedem Land ein Sachverständigen-Gremium unter Berücksichtigung der landesüblichen Kost oder anderer (z. B. klimatischer) Erwägungen über ihre Zulässigkeit entscheiden.

Es sei versucht, unser gegenwärtiges Wissen über das Verhalten der wichtigsten phenolischen Antioxydantien im Stoffwechsel in einigen Worten zusammenzufassen. Das Verhalten des BHA wurde an verschiedenen Tierarten und auch am Menschen untersucht. Bei der Ratte und beim Kaninchen wird es ohne Schwierigkeiten resorbiert und der größte Teil nach kurzer Zeit durch den Harn wieder ausgeschieden, und zwar direkt gekuppelt mit Glucuronsäure, in geringerem Maße mit Schwefelsäure. Bei Ratten wird außerdem ein kleiner Teil entmethyliert, so daß es zur Bildung des entsprechenden Phenols kommt. Der Umsatz im menschlichen Stoffwechsel erfolgt in ähnlicher Weise wie bei der Ratte und beim Kaninchen, nur wurde bis jetzt keine Entmethylierung festgestellt. Beim Hund weicht dagegen die Verarbeitung im Stoffwechsel von der eben geschilderten Verhaltensweise wesentlich ab. Anscheinend wird weniger als die Hälfte aus dem Darm resorbiert und der größte Teil derselben nach Kupplung mit Schwefelsäure ausgeschieden. Daneben wurde eine beachtliche Entmethylierung und Hydroxylierung des Moleküls festgestellt. Aus diesen Ergebnissen zog man den Schluß, daß sich der Hund als Versuchstier für toxikologische Untersuchungen zwecks Bestimmung der für den Menschen festzusetzenden BHA-Toleranzwerte weniger eignet als die Ratte oder das Kaninchen.

Erlaubte Tagesdosen von Antioxydantien beim Menschen

	tägliche Dosis pro Körpergewicht [mg/kg]	
Phenolisches Antioxydans	absolut	bedingt
Butyliertes Hydroxyanisol (BHA)	0—0.5	0.5—2
Butyliertes Hydroxytoluol (BHT)	0—0.5	0.5—2
Propylgallat	0—0.2	0.2—0.5
Octylgallat	0—0.2	0.2—0.5
Dodecylgallat	0—0.2	0.2—0.5
Tocopherol (Vitamin E)	0—1	1 —2

Auszug aus dem technischen Bericht der Weltgesundheitsorganisation Nr. 228, 1962, modifiziert 1965

Auch das BHT wird aus dem Darm leicht resorbiert, doch steht anscheinend bei diesem Stoff keine phenolische Hydroxylgruppe für die Kupplung zur Verfügung, wahrscheinlich wegen der benachbarten tertiären Butylgruppen. Im Kaninchen wird die Ringmethylgruppe zur Carbonsäure oxydiert, die sodann nach Kupplung mit Glucuronsäure und Glycin ausgeschieden wird. Es wird auch das Glucuronsäure-Derivat eines Alkohols ausgeschieden, der wahrscheinlich durch Oxydation einer tertiären Butylgruppe entsteht. An der Ratte haben wir nachgewiesen, daß nur wenig Alkohol ausgeschieden wird und daß der Hauptumsatzweg zu einer Carbonsäure durch Oxydation der Ringmethylgruppe führt. Der Hauptmetabolit im Menschen ist das Glucuronid eines Oxydationsproduktes des BHT und bisher noch nicht näher identifiziert. Dazu kommt wie bei der Ratte eine relativ kleine Menge einer Carbonsäure, und es ist wahrscheinlich, daß der enterohepatische Kreislauf, den wir bei der Ratte beobachtet haben, von der Gegenwart dieser Säure abhängt.

Von den drei Gallussäure-Estern wurde das Propylgallat am eingehendsten untersucht. Diese Verbindung wird zu freier Gallussäure hydrolysiert, die dann an der 4-Hydroxyl-Gruppe methyliert und — mit Glucuronsäure gekuppelt — das Hauptstoffwechselprodukt im Urin ergibt.

Das Tocopherol oder Vitamin E ist ein sehr komplex aufgebautes substituiertes Phenol. Der Mechanismus der Resorption dieses Stoffes aus dem Verdauungstrakt ist recht ungewöhnlich, wahrscheinlich weil es sich um einen wesentlichen Bestandteil der Nahrung handelt. Es dauert lange, bis eine Einzeldosis ausgeschieden wird; schließlich erscheint der Stoff im Harn, aber in einer Form, die keine Vitamin E-Aktivität mehr aufweist. Soviel mir bekannt, ist noch nicht geklärt worden, wie die Umwandlung dieses Stoffes im Organismus vor sich geht.

Antioxydantien werden auch weitgehend verwendet, um die Autoxydierung von Fetten in Futtermitteln zu verhindern. Es geht hier nicht nur um die Gesundheit des Tieres, sondern auch darum, eine Kontaminierung der für den menschlichen Gebrauch bestimmten Produkte (Fleisch, Eier, Molkereiprodukte) zu verhüten. Die wichtigsten phenolischen Antioxydantien, die zu diesem Zweck verwendet werden, sind das BHT und das BHA. Einschlägige Untersuchungen haben ergeben, daß eine solche Behandlung der Futtermittel die menschliche Gesundheit in keiner Weise gefährdet.

Phenolische Antioxydantien haben noch einen weiteren Verwendungszweck: Sie dienen dazu, die Haltbarkeit von Polyolefin-Kunststoffen zu verbessern. Wenn nun solche Kunststoffe bei der Verpackung von Lebensmitteln Verwendung finden, besteht die Möglichkeit, daß während der Lagerung das Antioxydans aus der Verpackung in die Lebensmittel übergeht. Für Verpackungszwecke werden allerdings meistens Antioxydantien mit hohem Molekulargewicht verwendet, nicht nur um diese Art von Wanderung zu verringern, sondern auch, damit sich das Antioxydans bei der Wärmebehandlung des Kunststoffes nicht verflüchtigt. Mehrere dieser Zusätze wurden in Tierversuchen untersucht, wobei es sich zeigte, daß nur sehr geringe Mengen aus dem Darm resorbiert werden.

Zusammenfassend läßt sich feststellen, daß die Untersuchung des Verhaltens chemischer Stoffe im menschlichen und tierischen Stoffwechsel interessante Einblicke in die Mechanismen bietet, mit denen der Organismus Fremdstoffe, die mit der Nahrung aufgenommen werden, „neutralisiert". Diese biochemischen Untersuchungen können auch wertvolle Aufschlüsse darüber geben, welche Stoffe als Lebensmittelzusätze Verwendung finden können, ohne die menschliche Gesundheit zu gefährden.

räumlich behindert wird, mit Glucuronsäure zu Aryl-Glucosiduronaten oder mit Schwefelsäure zu Monoestersulfaten gekoppelt. Ist keine reagierende Hydroxylgruppe vorhanden, so kann der Ring durch Mikrosomenoxydase hydroxyliert werden, genauso wie dies bei der Umwandlung von Benzol in Phenol geschieht. Eine Methoxy-Gruppe kann bei Anwesenheit gewisser anderer Ringsubstituenten zum entsprechenden Phenol entmethyliert werden. Alkylgruppen können zu Carboxylgruppen oxydiert werden. So entsteht aus Toluol Benzoesäure. Die Säure wird dann entweder mit Glucuronsäure zu einem Aryl-glucosiduronat (Esterglucuronid) oder mit Glycin zu einem Hippursäurederivat gekoppelt. Die Stoffwechselenzyme können aber auch Veränderungen bewirken, die die Löslichmachung und Ausscheidung aus dem Organismus hemmen; phenolische Hydroxylgruppen können methyliert oder Carboxylgruppen durch Eliminierung von Kohlensäure zerstört werden. Wo der Umsatz im Stoffwechsel auf mehr als einem Weg vor sich gehen kann, hängt das Mengenverhältnis der Endprodukte von reaktionskinetischen Faktoren ab, die bei verschiedenen Tierarten, aber auch bei Tieren der gleichen Art nach Geschlecht, Alter, Ernährung und vielleicht auch nach „Stress" unterschiedlich sein können.

Bis vor etwa zehn Jahren wurden im Rahmen experimenteller Untersuchungen des Stoffwechsels neuer Verbindungen den Versuchstieren, meistens Ratten, Kaninchen oder Hunden, große Einzeldosen oder mehrere kleinere Dosen gegeben. Sodann wurde der Urin gesammelt und durch geeignete Verfahren fraktioniert, um die Stoffwechselprodukte in reinem Zustand isolieren und nach Schmelzpunkt, Elementaranalyse usw. charakterisieren zu können. Bei solchen Untersuchungen gelang es meist nur, die wichtigsten Stoffwechselprodukte zu identifizieren, und wenn einmal mehr als 50 % des ursprünglich verabreichten Stoffes in Form von Derivaten aus dem Urin zurückgewonnen werden konnten, so war dies schon ein außerordentlicher Erfolg. In letzter Zeit wurden diese Arbeiten durch die Einführung radioaktiver Indikatoren wesentlich erleichtert. Der zu prüfende Stoff wird an einer geeigneten Stelle des Moleküls mit einem schwachen β-Strahler, meist C^{14} oder Tritium, synthetisiert und sodann in kleineren Dosen verabreicht, die den in der Nahrung vorhandenen Mengen in etwa entsprechen. Die radiochemischen Methoden sind so empfindlich, daß man mit radioaktiven Dosen arbeiten kann, die für Tiere — und auch für Menschen — völlig ungefährlich sind. Der erste Schritt ist die Untersuchung der Radioaktivitätskurven der Exkremente und eventuell auch der ausgeatmeten Luft und die Aufstellung einer „Bilanz" der verabreichten, der ausgeschiedenen und der im Gewebe aufgespeicherten Menge. Es kann auch die Ausscheidung durch die Galle in den Darm untersucht werden, indem eine Kanüle in den Gallengang eingeführt wird.

Ein anschauliches Beispiel für den Wert radiochemischer Verfahren bieten die vor kurzem in unseren Laboratorien durchgeführten Untersuchungen über den Stoffwechsel von 2,6-Di-tert.-butyl-4-methylphenol (butyliertes Hydroxytoluol, BHT). In früheren Arbeiten konnte im Urin nur ein relativ geringer Teil einer peroral verabreichten Dosis und im Kot überhaupt keine Spur festgestellt werden. Man schloß auf Gewebespeicherung, eine Annahme, die ernstliche Zweifel an der Eignung dieses Stoffes als Lebensmittelzusatz aufkommen ließ. Unter Verwendung chemischer Methoden zeigten wir aber, daß Ratten, die mit BHT-haltiger Kost gefüttert wurden, nur wenig BHT im Körperfett und in der Leber enthielten und daß auch diese geringfügigen Mengen aus diesen Organen nach kurzer Zeit wieder verschwanden, wenn die Ratten wieder auf Normalkost gesetzt wurden. Wir verabreichten den Ratten sodann eine Dosis des BHT, das an den tertiären Butyl-Gruppen mit C^{14} markiert war. Auf diese Weise gelang es uns innerhalb von vier Tagen bis zu 90 % aus den Ausscheidungen zurückzugewinnen, wobei ein großer Teil auf den Kot entfiel. Eine Untersuchung der Gewebe, die 2 bzw. 7 Tage nach der Verabreichung durchgeführt wurde, ergab eine nur schwache Radioaktivität, und die Zeit, in der das BHT zur Hälfte aus dem Organismus ausgeschieden worden war, wurde auf weniger als 2 Tage geschätzt. Am stärksten radioaktiv war der Darm. Da, wie wir bereits nachgewiesen hatten, die verabreichte Dosis zum Großteil durch die Galle ausgeschieden wird, dürfte die Erklärung darin liegen, daß die Ausscheidung des BHT im inneren Kreislauf zwischen Leber und Darm etwas verzögert wird, indem die mit der Galle in den Darm ausgeschiedenen BHT-Derivate zum Teil resorbiert und in die Leber zurückgeleitet werden, von wo sie zum zweiten Male in den Darm gelangen. Versuche, in denen C^{14} markiertes BHT dem Menschen verabreicht wurde, haben allerdings keinen Hinweis auf einen solchen enterohepatischen Kreislauf ergeben. Mehr als 50 % der Dosis konnten im Urin innerhalb von 24 Std. festgestellt werden; am zweiten Tag fiel die Ausscheidung merklich ab und war dann weiterhin für mehrere Wochen in immer verringertem Maße vorhanden. Ein ähnliches Antioxydans, 3-Tert.-butyl-4-hydroxyanisol (butyliertes Hydroxyanisol, BHA) wird im Urin des Tieres und des Menschen zum größten Teil innerhalb 24 Std. ausgeschieden. Beim Menschen folgt dann dieser erstmaligen Ausscheidung eine langwährende geringe Ausscheidung im Urin, wie wir sie mit BHT beobachtet haben. Es ist möglich, daß bei beiden Antioxydantien diese verzögerte Ausscheidung das Freiwerden kleiner Mengen von Metaboliten darstellt, die in den Geweben gespeichert worden sind.

Auch bei der qualitativen Untersuchung metabolischer Prozesse werden heute weitgehend radioaktive Isotopen verwendet. Der Urin oder auch andere biologische Substanzen werden nach verschiedenen Verfahren fraktioniert, meist durch organische Lösungsmittel (Flüssigkeitsextraktion), Ionenaustauschharze und Säulenchromatographie, und die Anwesenheit von Stoffwechselprodukten in jeder Phase durch Auswertungen der Radioaktivität in den verschiedenen Fraktionen ermittelt. Zur weiteren Isolierung und Abtrennung der Stoffwechselprodukte bedient man sich heute mit Vorliebe der Dünnschichtchromatographie, wobei die radioaktiven Punkte durch Autoradiographie lokalisiert werden. Die einzelnen Stoffwechselprodukte können auf infrarot-spektroskopischem oder massenspektrometrischem Wege oder auch durch Messungen der kernmagnetischen Resonanz bestimmt werden. Zur Bestätigung des Bestimmungsergebnisses wird schließlich ein Stoff der gleichen hypothetischen Struktur auf eindeutigem Weg synthetisch hergestellt und mit dem natürlichen Stoffwechselprodukt unter Anwendung geeigneter chemisch-physikalischer Methoden verglichen.

Die Verwendung radioaktiver Indikatoren hat zwar einerseits die Bestimmung der Hauptprodukte des Stoffwechsels beträchtlich erleichtert. Andererseits aber gibt sie uns wieder neue Probleme auf, weil wir jetzt mit ihrer Hilfe viele andere Produkte feststellen, die in geringeren Mengen vorkommen und früher leicht übersehen wurden. So werden zum Beispiel bei der Oxydation einer Methylgruppe zu einer Carboxylgruppe zuweilen geringe Mengen des entsprechenden Zwischenalkohols und Aldehyds und deren Derivate ausgeschieden.

In Deutschland ist zur Zeit das Tocopherol (Vitamin E) das einzige Antioxydans, das allein oder in Kombination mit Synergisten Speisefetten zugesetzt werden darf. In

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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

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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

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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

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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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Fate of Butylated Hydroxyanisole in Man and Dog

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To see if results in rats, rabbits, and dogs at high dose levels justified extrapolation, the fate of butylated hydroxyanisole in man was studied. Further possible pathways were sought in dogs. Dogs excreted 60% of a 350 mg. per kg. dose unchanged in the feces within 3 days, and the remainder in the urine largely as sulfate conjugates of BHA, *tert*-butylhydroquinone, and an unidentified phenol. Little glucuronide was formed. Procedures used for low dose levels in rats were applied to man, where 0.5 to 0.7 mg. per kg. yields in the urine less than 1% of the dose as unchanged BHA, and 27 to 77% as the glucuronide of BHA, mostly within 24 hours. No dealkylation or hydroxylation was found. Small doses in man and large doses in rats and rabbits have similar fates, and extrapolation from these species is justified. Comparative metabolism studies are important for safety evaluation of additives, particularly if extension to man is permissible.

BUTYLATED HYDROXYANISOLE, BHA, a mixture of more than 96% of 3- and less than 4% of 2-*tert*-butyl-4-methoxyphenols, was introduced as a fat stabilizer about ten years ago. It has found general use in a variety of products, and its use as an additive in foods has been accompanied by a number of studies of its safety. Acute and chronic studies have shown BHA to have a low toxicity in dogs (*6*, *9*) and rats (*8*). No adverse effects on humans have been reported. Prolonged dosage of rats at several diet levels caused no detectable storage in the perirenal, omental, or subcutaneous fat (*8*).

The metabolic fate of several antioxidants, including BHA, has recently been reviewed (*1*), and studies on the fate of BHA in the rabbit (*5*), dog (*9*), and rat (*1*) have been reported. In the rabbit, an oral 0.5-gram dose was excreted as glucuronide (60%), ethereal sulfate (12%), and uncombined phenols (4%), the constituent isomers being metabolized similarly. In the dog, elevated glucuronic acid output and ethereal sulfate ratios accompanied repeated daily doses of 0.25 gram per kg. In the rat, single doses of 0.4 gram per kg. were accounted for as glucuronide (61 to 82%), ethereal sulfate (11 to 16%), and unchanged BHA (5%). The 3-*tert*-butyl isomer underwent conversion mostly to its glucuronide in the rat, whereas the 2-*tert*-butyl isomer formed mostly a sulfate, and a small proportion seemed to be demethylated (*1*).

No metabolic studies of BHA in man have been reported. Extrapolation to man from animal studies appears to involve several unknowns. Species differences involve differences in metabolic pathways. The dosages used in these studies are large compared with the estimated human intake, which is probably less than 0.1 mg. per kg. Large doses in experimental studies generally produce stress clearly absent with somewhat smaller doses and may also cause the use of metabolic pathways unused with small doses. The order of ethereal sulfate conjugation varies with dose level (*3*), so that the relative proportions of metabolites formed may vary with the size of the dose. A similar result, involving in addition any unmetabolized fraction of the dose, may follow where any of the rates of conjugation, excretion, and renal tubular secretion or excretion are related to the dose level. Therefore, information about the metabolism of a substance gained from large dose studies may be misleading.

We have reported a study of variation in metabolic pathway with dose in the rat (*1*). At dose levels of from 100 to 2 mg. per kg., a fairly close correspondence with metabolism at the large dose level was found, with 81 to 100% recovery of the dose. At low dose levels the proportion of the dose excreted unaltered in the urine increased somewhat, possibly because rates of excretion and conjugation became comparable. The time required for complete excretion of the dose decreased as the dose decreased, and the time for glucuronide excretion at the lowest level was of the same order as that for the unchanged BHA.

Since the ultimate use of BHA is in man, we judged it essential to apply the procedures developed with rats to man, and report the results in this paper. As a guide to the detection in man of further possible metabolic pathways, the fate of BHA in dogs was also studied.

Procedures

Dosages, Diets, Methods. DOGS. Three male mongrel dogs of about 14-kg. weight, fed Purina Dog Chow, were kept singly in metabolism cages. Some difficulty was encountered in feeding large single doses of BHA, but doses of about 5.0 grams (0.35 gram per kg.) were given as 7.5% (w./w.) mixtures in lard, subsequently mixed with the daily diet. Urines were collected after dosing until no further metabolites were detectable; feces were collected for the same period and pooled for each dog. Control values for metabolites were established by analysis of urines collected for a few days before dosing. Excreta were analyzed immediately or kept frozen until analysis.

HUMANS. Male adult volunteers from this department received single oral doses of BHA, given as the crystalline powder (50 mg.) in a gelatin capsule, or as a homogenate in milk–olive oil (100 to 1 by volume). After mixing, the homogenate was decanted and sufficient ingested to give about a 30-mg. dose. A sample of the material ingested was assayed by continuous ether extraction, followed by spectrophotometric estimation of the ether-extracted BHA (*7*). Urines were collected until no metabolites were detectable. Each voiding was kept separate and where possible refrigerated. Control values for each subject were established before dosing.

BHA was detected after chromatography in the solvent systems (Table I), and was estimated densitometrically, after spraying with Gibbs' reagent (2,6-dichloroquinonechlorimide) and a 2% borate solution, at 595 mμ by the procedure previously described (*1*). Whatman No. 1 filter paper was used throughout in the descending technique.

Examination of Dog Urine. Total and inorganic sulfate content was determined by Folin's gravimetric method (*2*). The glucuronide of BHA was estimated by the enzymic hydrolysis procedure used for low dose studies (*1*), as normal glucuronide excretions were too large and the changes in level too small for the naphthoresorcinol method to be used. Unchanged BHA was determined chromatographically and densitometrically (*1*). Control and experimental urines (10 or 20 μl.) were chromatographed to detect urinary BHA or any chromogenic metabolites (*1*). Solvent systems and spray reagents are listed in Table I.

Examination of Dog Feces. The collection from dosing experiments or an aliquot of control feces was exhaustively extracted (Soxhlet) for 48 hours with benzene. BHA in the benzene extract was identified and estimated chromatographically using Gibbs' reagent (*1*). Attempts to isolate BHA from the extract by steam or vacuum distillation resulted in very impure oils of high BHA content.

Processing of Dog Urine for Metabolites of BHA. Chromatographic examination of daily urines revealed that one, and probably two, chromogenic substances, compounds I and J, absent from control urines, occurred in urines following the ingestion of single large doses of BHA (Table I). The maximum detectable amounts occurred on the first and second days after dosing; by the fourth or fifth day, no metabolites were detectable. Free BHA could not be detected chromatographically in experimental urines.

Urine (2000 ml.) from two dogs, each receiving a 5-gram dose, collected on the three days following the dose, was pooled and the acid and alkaline glucuronide gums were prepared as previously described (*1*) by lead salt precipitation. Both gums contained small amounts of the glucuronide of BHA, as shown by the liberation of BHA by treatment of the gum with β-glucuronidase (*1*). Chromatographic examination showed that the alkaline gum was considerably enriched with two substances absent from a control gum (compounds I and J, Table I); these were considered to be conjugates of metabolites of BHA, but not glucuronides, since they were unaffected by β-glucuronidase.

The possibility that the two metabolites were ethereal sulfates led to the following procedure for their isolation.

The alkaline gum was dissolved in H_2O (100 ml.) and neutralized with solid K_2CO_3. An inorganic precipitate was filtered out and the solution evaporated to a gum. This was repeatedly extracted with warm 90% aqueous (v./v.) 2-propanol (total, 1 liter), followed by hot absolute ethanol (total, 700 ml.) until in each case no further metabolites were extracted. The combined solvents yielded a gum, to the aqueous solution (30 ml.) of which was added safranine bluish (Distillation Products Industries, Rochester, N. Y.) (5.0 gram) in water (100 ml.). The precipitated phenazinium salts of the sulfate conjugates (*1*) were collected the next day, and after aqueous washing yielded a brown-green solid (4.2 grams). This refused crystallization and the phenazinium ion was exchanged for K^+, using Dowex 50 (K^+ form) (*1*). The aqueous solution of potassium salts was evaporated to a small semisolid residue which refused crystallization. Chromatography showed the residue to be largely compounds I and J. It was warmed with 3*N* HCl (20 ml.) for 20 minutes at 60°, cooled, and extracted with ether (3 × 50 ml.).

The aqueous solution gave a strong positive SO_4^{-2} reaction, and the ethereal solution, on drying ($MgSO_4$) and evaporation, gave a small amount of a yellow oil. Chromatography showed this to contain three phenols (A, B, and C, Table I). A was shown chromatographically to be BHA, and B had the constants and reactions of *tert*-butylhydroquinone.

Attempts to crystallize this oil or fractionate it with solvents failed, and it was partitioned on a previously equilibrated column of powdered filter paper (Whatman No. 1) with benzene–acetic acid–water (2:1:1 by volume; organic phase). Fractions of 5 ml. were collected automatically and combined on the basis of maximum absorption at 290 mμ into fractions I (5-ml. fractions 5 to 11), II (13 to 15), and III (41 to 59). BHA and a trace of phenol C were located chromatographically in fraction I, *tert*-butylhydroquinone in II. III contained no metabolites and was discarded.

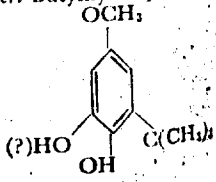
Fraction II was evaporated in vacuo to a crystalline residue, which on solution in hexane slowly deposited needles

Table I. Chromatography of Ethereal Sulfate Conjugates of BHA and Its Metabolites in the Dog

Solvents
- I. Organic phase of freshly mixed butanol–acetic acid–water (4:1:5 by vol.)
- II. Organic phase of benzene–acetic acid–water (2:2:1 by vol.)

Spray Reagents
- A. Aq. 1% (w./v.) $AgNO_3$ mixed with 1 part aq. 3*N* NH_4OH
- B. Ethanolic 0.05% (w./v.) 2,6-dichloroquinonechlorimide; the dried paper was sprayed with aq. 2% (w./v.) sodium borate, or
- C. With aq. 10% (w./v.) Na_2CO_3
- D. Equal volumes, freshly mixed, of 1% (w./v.) *p*-nitraniline in 3*N* HCl and aq. 5% (w./v.) $NaNO_2$. The dried paper was sprayed with aq. 2% (w./v.) Na_2CO_3.

Source	Substance	Solvent Systems, R_f Values I	Solvent Systems, R_f Values II	Colors Given by Reagents[a] A	B	C	D	Assigned Structure
Urine after 350 mg./kg. dose.	Compound I	0.85	0.00	0	Blue-purple	Blue	Orange	O-Sulfate conjugates of phenols B and C
	Compound J	0.75	0.00	0	0	Purple	Red-purple	
Ether extract of hydrolyzate of potassium salts of ethereal sulfate conjugates	Phenol A	0.96	0.94	Blue-brown	Blue	Purple	Orange	BHA
	Phenol B	0.96	0.55	Black	Blue-purple	Purple	Orange	*tert*-Butylhydroquinone
	Phenol C	0.96	0.80	Black	Blue-purple	Purple	Orange-red	



[a] With aq. 2% (w./v.) $FeCl_3$ solution, by phenol C only, yellow, turning to emerald green with *N* Na_2CO_3.

(22 mg.) of *tert*-butylhydroquinone (m.p. 126–9°) undepressed on admixture and having ultraviolet and infrared spectra identical with an authentic specimen. Calculated for $C_{10}H_{14}O_2$: C, 72.2; H, 8.5%. Found: C, 71.9; H, 8.4%.

Attempts to isolate phenol C from either fraction I or other hydrolyzates and fractions were unsuccessful and this substance appears to be largely destroyed on attempted separation. The urine remaining after precipitation of the glucuronide fractions was deleaded and found to contain only trace amounts of compounds I and J; it was discarded. Portions of both glucuronide gums were incubated with β-glucuronidase (*1*) and the hydrolyzates extracted with ether. BHA was detected, but no phenols derived from possible conjugates of BHA metabolites were found. Because of the small quantity of BHA glucuronide present, it was not possible to isolate it.

A portion of the ethereal sulfate of the major isomer of BHA, prepared as previously described (*1*), was submitted to the hydrolytic procedure described above. BHA was the only detectable phenolic product, thus excluding the possibility that phenol C and *tert*-butylhydroquinone were artifacts of BHA or its sulfate.

Examination of Human Urines. Experimental and control urines were analyzed for unchanged BHA and its glucuronide by procedures essentially similar to those described for low dose studies in the rat (*1*). The glucuronide estimation was modified to diminish interference from normally occurring phenolic glucuronides as follows.

The enzymic hydrolysis was performed by mixing 15 ml. of 0.2*M* pH 4.8 phosphate buffer, the corresponding glucuronide fraction from 10 ml. of human urine, and 7 ml. of β-glucuronidase solution. After incubation, the mixture was twice extracted with ligroin (50 ml., b.p. 35–60°). The extracts were centrifuged to de-emulsify them and washed with 25 ml. of aqueous 7% (w./v.) $NaHCO_3$, the alkaline extract was washed with ligroin (50 ml.), and the combined ligroin extracts were cautiously evaporated on a steam bath.

In this way phenolic acids having chromatographic properties identical to those of BHA were prevented from interfering with the final densitometric determination of the BHA content of the residue (*1*).

Results

The average control values (expressed as BHA) for daily excretions by the three dogs were: free phenols (six determinations) 1.9 (1.0 to 2.3) mg.; glucuronides (six determinations by enzymic procedure) 5.2 (3.8 to 6.0) mg.; ethereal sulfates (14 values) 0.32 (0.2 to 0.49) gram, ethereal–inorganic sulfate = 0.230. Normal daily excretions by humans, in 12 determinations on eight subjects, as BHA were: free <1mg.; of the glucuronide of BHA, 4.0 (2.5 to 5.5) mg.

A study with 350 mg. per kg. doses in dogs showed that most of the dose was readily recovered from the excreta, but several differences from the metabolism pathway in rats and rabbits were found (Table II). A large proportion of the dose is excreted by the dog unaltered in the feces. Glucuronide conjugation is not the main metabolic pathway, but a preponderant sulfate conjugation of the absorbed material is found. Separation of the sulfate conjugate by the phenazinium salt method used for the rat and subsequent ion exchange and mild hydrolysis of the potassium salts showed the elevated sulfate level to be caused partly by the formation of the 4-*O*-sulfate of BHA and two further sulfate conjugates. The isolation of *tert*-butylhydroquinone, derived from one of these, establishes *O*-demethylation as a significant metabolic pathway for BHA in the dog. The other conjugate yielded a phenolic substance whose color reactions on paper chromatograms are consistent with its being a nuclear hydroxylation product of BHA. About the same proportion of the dose is excreted in the urine unchanged as in the rabbit and rat, and the glucuronide conjugate, as in those species, appears to be exclusively with the 4-hydroxyl of BHA.

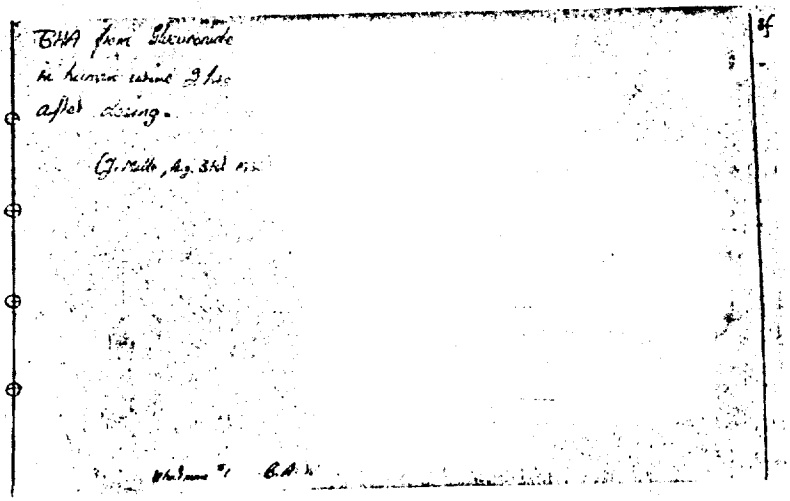


Figure 1. Chromatograms of BHA

Upper. 20 μg. of BHA
Center. Two human subjects each receiving 30 mg. of BHA; spots are BHA derived from its glucuronide
Lower. Human control urine
Spray reagent. B (Table I). BHA spots, blue-purple. Control spot, weak brown-green

Table II. Excretion of BHA by Dogs

(Mean % recoveries of a 350 mg. per kg. dose, with ranges in parentheses)

BHA	Days after Dosing 1	2	3	4	Totals
Urinary					
Free	1.1(0.7–1.6)	2.3(1.2–3.0)	0.2(0–2)	0	3.6
Ethereal sulfate	9(1–20)	11(8–18)	2(0–3)	1(0–4)[a]	23.0
Glucuronide[a]	4.0(1.8–8.1)	1.2(0.6–1.8)	0.3(0.2–0.4)	0	5.5
Fecal					
Unchanged[b]					51, 62

[a] Enzymic method (*1*) for small doses.
[b] Determined on 2 dogs.

Table III. Urinary Excretion of BHA by Man

(Free BHA was invariably less than 1% of dose)

Medium	Average Dose, Mg.	Subject	BHA as Glucuronide, % Recovery	Period for Excretion of Glucuronide, Hours
Olive oil–milk homogenate	31	A	77	23
		B	51	23
		C	39	31
		B	36	27
Capsule	50	D	71	23
		B	41	50
		E	27	38
		F	32	27

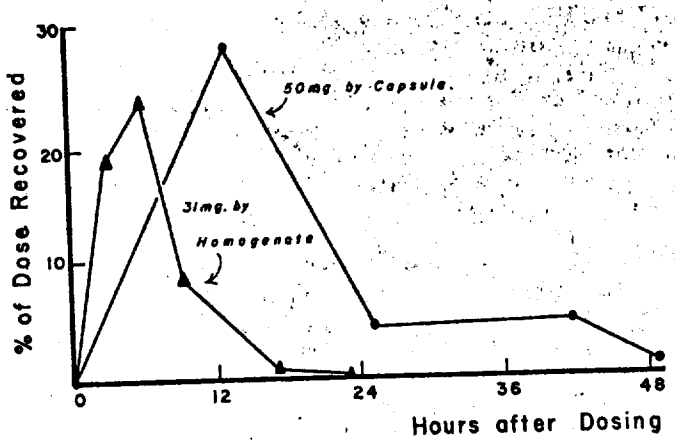


Figure 2. Urinary excretion of BHA as a glucuronide by man

Urines voided at times indicated after dosing.
● slowest and ▲ most rapid excretions of BHA glucuronide at dose levels shown

Table IV. Metabolism of BHA

(% of single dose given)

Species	Rabbit (5)	Rat (1)		Dog	Man
Dose, mg./kg.	500	400	2	350	0.4–0.7
Unabsorbed		0–3		56	?
Unconjugated	4	5	10.3	3.6	<1
Ethereal SO_4	12	14		23	Yes (?)
Glucuronide	60	72	72	5.5	27–77
Demethylated		Yes[a]		Yes	No
Hydroxylated		No		Yes (?)	No

[a] Minor metabolite.

In applying the procedures for low doses in rats to man, we found that recoveries of added free BHA from human urine were 90 ± 10%, and of the glucuronide of BHA, less satisfactory than in the rat, 50 ± 7%. BHA in human urine was detectable at concentrations of 1 μg. per ml., and the background absorption on chromatograms, caused by naturally occurring chromogens similar in properties to BHA, was less than 1 μg. per ml. The normally occurring phenolic glucuronides in human urine yielded on enzymic hydrolysis a variety of phenols and phenolic acids. Some of these gave background values on chromatograms where BHA was located of the same order as that expected to be given by BHA at a dose level of 50 mg. A variety of solvent systems was tried without effectively reducing this value. However, the use of ligroin as an extracting solvent, and especially the removal of phenolic acids from the ligroin extract of the hydrolyzate with an alkaline wash, diminished the background.

The average background value for normal phenols and for the glucuronide excretion recorded in Table III was taken as 8 ± 3% of a 50 mg. per kg. dose as BHA. At the levels of dosing recorded, the limits of specificity and precision have been reached in the glucuronide estimation. The lower limit of detectability of the glucuronide was of the order of 2 μg. per ml. of urine.

Table III records the results of analyses of urine from volunteers receiving BHA in each form. Figure 1 shows a chromatogram in which a standard quantity (20 μg). of BHA, BHA derived from its glucuronide in two experimental urines within a short while of dosing, and a control hydrolyzate, are co-chromatographed in solvent I, Table I. Small discrepancies in R_f values of BHA in experimental urines are usual in solvent I, and may be caused by varying urinary constituents. BHA undoubtedly forms a glucuronide in man and, by analogy, probably with its phenolic hydroxyl. Subject to the limits discussed above, 27 to 77% of the dose is excreted in the urine in this form. Urines were also examined for other metabolic pathways, but no demethylated or hydroxylated products could be detected. The possibility of ethereal sulfate formation exists, as dilute acid hydrolysis of some experimental urines occasionally liberated BHA. Free BHA was not detectable in human urines and no account could be made of the remaining nonconjugated BHA However, absorbed BHA is rapidly metabolized, as Figure 2 shows, where the rates of fastest and slowest excretion for the indicated doses of BHA as a glucuronide are recorded. All other rates of excretion lie within these values. Maximum excretion usually occurs within 17 hours of the dose, and excretion of glucuronide is complete by the second day after ingestion. Refrigeration and immediate analysis, when possible, of collections in the human study were important in obtaining high BHA glucuronide values. Where analysis had to be delayed, glucuronide values were frequently low.

Discussion and Conclusions

As an adjunct to toxicity testing in the safety evaluation of an additive, knowledge of metabolic pathways can be of particular value. Thus, once the existence of absorption has been shown, retention periods within the organism, the likelihood of tissue storage, and whether the absorbed material is converted to normally present metabolites or is metabolized in a fashion usual for the class of compound studied can be ascertained. However, since such results usually result from animal studies only, there is no guarantee of their applicability to man.

In the case of BHA, the theoretically possible metabolic pathways after absorption include conjugation at the phenolic hydroxyl, nuclear hydroxylation and *O*-demethylation, oxidation of the side chain $C(CH_3)_3 \rightarrow C(CH_3)_2.CH_2OH$, and conjugation of the metabolites. Table IV presents the metabolic fate of BHA for all the species so far reported. Without confirmatory evidence in man, no *a priori* reason existed for extrapolating from the results in any one of these species, and a wider spectrum of species widens the choice of any or all of the postulated pathways. It is gratifying to find that a major metabolic pathway in rats and rabbits is also common to man. Within the limitations of the methods of analysis, the great difference between the dose level, in most of the animal studies and the level in man, does not lead to large discrepancies in the manner in which BHA is metabolized.

In this instance, the choice of the dog as the sole experimental animal would have led to highly misleading results. The high level of nonabsorption would have caused speculation about complete nonabsorption at low levels of dosage, and the major metabolic pathway differs from the other species studied. This study illustrates the value of comparative studies, both in species and at various dose levels, in metabolism studies as a part of safety evaluation. The value is enhanced when analytical techniques permit a study in the subject for which safety is being evaluated—i.e., man—of dose levels near those encountered in use.

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ALFALFA HEMICELLULOSE

Constitution of the Hemicellulose of Alfalfa (*Medicago sativa*). Further Studies on the Acidic Components Produced by Hydrolysis

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The constitution of the hemicellulose of alfalfa (*Medicago sativa* var. Ranger) is being investigated in an attempt to ascertain the possible relationship between chemical structure and nutritional value for ruminants. Three aldobiouronic acids, 2-O-α-D-galactopyranosyluronic acid-L-rhamnose, 6-O-β-D-glucopyranosyluronic acid-D-galactose, and 2-O-α-D-glucopyranosyluronic acid-L-xylose, have been obtained by acid hydrolysis of alfalfa hemicellulose, in addition to the five acidic components and five neutral sugars reported previously. The results may be of value in ascertaining the role of hemicelluloses in animal nutrition.

THE HYDROLYSIS of the hemicellulose of alfalfa (*Medicago sativa* var. Ranger) leading to a mixture of neutral and acidic components was described in a previous communication (*7*). The neutral components were shown to be L-arabinose, D-xylose, D-galactose, D-glucose, and L-rhamnose. Of the seven acidic components encountered, five were identified as oxalic acid, D-galacturonic acid, 4-*O*-methyl-D-glucuronic acid, 2-*O*-(4-*O*-methyl-α-D-glucosyluronic acid)-D-xylose, and *O*-4-*O*-methyl-α-D-glucosyluronic acid-(1→2)-*O*-β-D-xylopyranosyl-(1→4)-β-D-xylose. The two other unidentified acids, which were thought to be galacturonic acid containing aldobiouronic acids, are shown herein to be 2-*O*-α-D-galactopyranosyluronic acid-L-rhamnose (I, R = H) and 6-*O*-β-D-glucopyranosyluronic acid-D-galactose (IV, R = H). Another acid, 2-*O*-α-D-glucopyranosyluronic acid-D-xylose (VIII, R = R_1 = H), which brings the total to eight, was derived from an aldotriouronic acid which appears to be 2-*O*-α-D-glucopyranosyluronic acid (1→2)-*O*-β-D-xylopyranosyl-(1→4)-D-xylose (VII), the analog of the previously identified aldotriouronic acid cited above which contained 4-*O*-methyl-D-glucuronic acid.

Experimental

All evaporations were carried out in vacuo at 35° to 45° C. (bath temperature) unless specified otherwise.

The ion exchange resins, Amberlite IR-120 (H^+ form) and Duolite A-4 (OH^- form), were used throughout.

The irrigating solvents used in chromatography were: *A*, *n*-butyl alcohol–acetic acid–water (2:1:1); *B*, ethyl acetate–acetic acid–formic acid–water (18:3:1:4); *C*, pyridine–ethyl acetate–water (1:2.5:3.5); *D*, *n*-butyl alcohol–ethyl alcohol–water (4:1:5); and *E*, butanone-water azeotrope.

Separation of Uronic Acid Components. The three uronic acids, 2-*O*-α-D-galactopyranosyluronic acid-L-rhamnose (I, R = H), 6-*O*-β-D-glucopyranosyluronic acid-D-galactose (IV, R = H), and an unknown aldotriouronic acid (?VII), which were separated from the other uronic acid components of alfalfa hemicellulose by cellulose column chromatography as described previously (*7*), had approximately the same R_f value (0.16) using solvent *A*. Examination showed that the aldotriouronic acid could be separated from the mixture of the two aldobiouronic acids by electrophoresis (*2*) using Whatman No. 1 paper, 0.1*M* borate buffer, and *p*-anisidine–phosphoric acid spray reagent. Separation on a preparative scale was carried out as follows: The sirup (0.40 gram) was applied to eight pieces of Whatman No. 3MM paper (5 × 22 inches), and each paper was placed on the electrophoresis apparatus (*2*) for 4 hours using 0.1*M* borate buffer, 600 volts and 30 to 40 ma.

After each of the papers had been dried, a strip was cut from the center and sprayed with the *p*-anisidine–phosphoric acid reagent to locate the position of each component. The components were isolated by extracting the appropriate section of the paper with water, and each solution was passed through the cation exchange resin. The effluent was evaporated to dryness. The residue was then treated for 30 minutes with 0.5% methanolic hydrogen chloride at room temperature after which the solvent was evaporated at room temperature to remove borate (*2*). In this manner an aldotriouronic acid (130 mg.), possibly α-D-glucopyranosyluronic acid-(1→2)-D-xylose-(1→4)-D-xylose (VII), and a mixture, *F*, (180 mg.) consisting of the two aldobiouronic acids (I and IV, R = H) were obtained.

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Fd Cosmet. Toxicol. Vol. 5, pp. 475–479. Pergamon Press 1967. Printed in Great Britain

Excretion of Butylated Hydroxytoluene (BHT) and Butylated Hydroxyanisole (BHA) by Man

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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 μC/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 μC/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

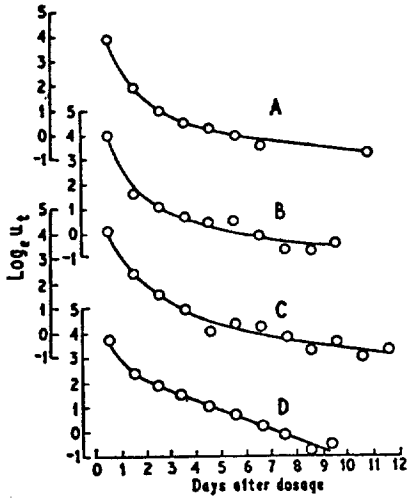

FIG. 1. Relationship between logarithm of daily excretion (u_t) and time. Subjects A and B received BHT; C and D, BHA.

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 excretés 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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Influence of the ingestion of antioxidants on the composition of certain tissues and on the stability of the reserve fats of pigs and chicken

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Summary

The effect of the ingestion of various antioxidants on their possible accumulation in muscle, reserve fats (dorsal and perinephric), liver and kidney, as well as on the resistance to oxidation of the reserve fats, has been studied in the pig and chicken.

The animals received in their ration either 0.125% diphenyl-p. phenylene diamine (DPPD), or 0.1%. butyl hydroxyanisol (BHA), butyl hydroxytoluene (BHT), dodecyl gallate, propyl gallate, or nordihydroguaiaretic acid (NDGA).

In the pig, only the DPPD is detectable in the reserve fats (maximum level found: 0.0025%), and in the kidney (maximum 0.0006%). In the chicken, DPPD also accumulates in the reserve fats, but the concentration is lower (0.0007%) than in the pig fat. BHT, however, is stored in chicken fat in an amount of 0.006%.

The study of the stability of the reserve fats of the control animals and of the treated animals confirms the result of the direct analysis of the antioxidants. In fact, the fats from pigs that have ingested DPPD and the fats of chicken that have received BHT exhibit a much longer induction period than that of the fats of control animals.

The technological and hygienic consequences of these observations are discussed.

Some natural antioxidants such as the tocopherols, normally contained in the diet of the animals, are able to increase the resistance to oxidation of the reserve fats. This fact was demonstrated in the rat (Barnes et al. 1943; Lundberg et al. 1944; Hanson et al. 1944; Burr, Lundberg and Chipault 1946;

Lundberg et al. 1947), the rabbit (Major and Watts, 1948), the pig (Carpenter and Lundberg 1949; Chipault, Lundberg and Burr, 1945; Watts, Cunha and Major, 1946), the chicken (Mecchi et al. 1956, a b; Hood, Wheeler and McGlamery, 1950), the turkey (Mecchi, Pool and Klose, 1953; Criddle and Morgan, 1951).

Research on other antioxidants, especially on synthetic products, is not very plentiful. When the present work was undertaken, in 1957, one of the few papers published dealt with the metabolism of diphenyl-p.-phenylenediamine in chicken (Pudelkiewicz et al. 1956). A little later, a short study on the pig was published (Lynch, Anderson and Lewis, 1958). Previously, Barnes et al. (1943) had shown that ingested hydroquinone, far from stabilizing the reserve fats of the rat, caused on the contrary an acceleration of the oxidation. So, the use of antioxidants in animal food is the necessary corollary of the incorporation of fats in foods. These substances can also be used to stabilize foods containing oxidizable fats such as fish meal. It is known, in fact, that the oxidation of the nutritional fats threatens to cause, in the animals which ingest them, nutritional accidents originating directly or indirectly from the presence of the products of oxidation. Therefore, to prevent some of these accidents such as encephalomalacia, some antioxidants are incorporated in the food ration of the animals.

The purpose of the present project is to study in the pig and chicken what are the consequences of the ingestion of various antioxidants on the composition of the animal tissues consumed by man.

MATERIAL AND METHODS

Antioxidants used - animals

The following antioxidants were studied: Propyl gallate; dodecyl gallate; butyl hydroxytoluene (BHT)(or dibutyl paracresol: DBPC); nordihydroguaiaretic acid (NDGA); butyl hydroxyanisole (BHA); diphenyl-para-phenylene diamine (DPPD). The dose added to the food was 0.1% for all products, except for the DPPD, which was added at the rate of 0.125%. This percentage corresponds to ten times that generally recommended for stabilizing food fats.

For the pigs, the period during which the diet enriched with antioxidants

was given was about four months (weight from 50 to 100 kg). Each lot, of which one was a control lot, comprised 6 animals. For the chicken, the test lasted eight weeks. Each lot comprised 15 birds.

Organs and tissues studied

In the pig, the dorsal fat (fatback) was taken off upon slaughtering. The two layers which constitute this tissue were separated and analyzed separately. Besides, the perinephric fat (porkfat) was taken. These fat tissues were rendered under CO_2 atmosphere, filtered and centrifuged. The following determinations were made on these fats: Amount of antioxidants, iodine number, peroxide number, stability test. The latter test was carried out in a lighted oven at 60°C, according to a method recommended by Desnuelle and by Wolff. The semi-tendinous muscle, liver and kidney were also sampled for analysis. In the chicken, the reserve fats were often absent, although we kept the animals as long as 16 weeks, in order to obtain sufficient fattening. Therefore we could not study the individual reserves. To obtain a sufficient quantity of fats, we had to combine the yields from two or three animals. For the muscle tissue, the pectoral muscles and the muscles of the leg were taken.

Quantitative analysis

Fats

The iodine numbers were determined by Wijs' method and the peroxide numbers (Lea) by iodometry.

Antioxidants

The method of Kahan (1954) with alpha-alpha' dipyridyl was used to determine the NDGA and the BHA. It was applied also to the quantiative analysis of the gallates. In fact, the ration of each of the lots contained only a single antioxidant, and consequently the lack of specificity of the reaction, which is an obstacle when dealing with a mixture, was no problem. It is necessary to trace a calibration curve for each series of determinations, by means of this reaction, regardless of the antioxidant analyzed. The readings were taken with the spectrophotometer at 515 m/u; the reaction time was 30 min for BHA and the gallates, 3 min for NDGA. The modification introduced by Anglin,

Mahon and Chapman was applied for the determination of the BHT (addition of n-butanol).

For the DPPD, the nitrogenous derivative was prepared, which is determined by colorimetry (Bunnel, 1954). In determining this antioxidant in the fats, we had to carry out a previous saponification, after having verified that this did not change the validity of the determination (5 g of fat, 2 g of KOH, 50 ml of alcohol, during a half hour at boiling temperature).

The extraction of the antioxidants from tissues and organs poses certain problems. In particular, the BHT must be entrained by the superheated steam, according to the method of Anglin, Mahon and Chapman (1956). We found, however, that the distillation temperature proposed by these authors was not suitable; it must be raised to 180°C in order to obtain a quantitative recovery of the antioxidant.

In the case of the fat tissues, these are dissolved in petroleum ether. The antioxidant (BHA, NDGA, gallates) is re-extracted with alcohol 72%. For the muscle, the extraction of the BHA and of the gallates occurs with the aid of petroleum ether, with the blender, by the method of Hanley (1954). Moreover, the presence of gallates in extremely low concentrations can be detected by addition of a few drops of concentrated ammonia (Wenger, 1954).

RESULTS

Validity of the analytical methods

Surcharge tests were carried out with the various antioxidants studied, to determine the lower limit of sensitivity, as well as the validity of the analysis. These surcharges were effected on the fat tissue, on the muscle, on the liver and on the kidney. The concentrations studied varied from 0.001% to 0.1%.

a) Fat

The recovery rates attain for several antioxidants values of the order of 95%, even for concentrations as low as 0.001%.

However, the recovery is lower (about 90%) with regard to the propyl gallate

and the BHT. In the latter case, the sequence of the operations of analysis, which comprises notably an entrainment with superheated steam, is probably responsible for this relatively low level. In no case has it been possible to confirm the results of Anglin, Mahon and Chapman. When strictly applying the method proposed by these authors, without changing the distillation temperature, the recovery rates did not exceed 50 to 60%.

b) Muscle and organs

The recoveries of the surcharges are also of the order of 95% as far as the muscle is concerned. As to the kidney and the liver, certain difficulties arise for the DPPD and the propyl gallate. We had to give up analyzing the BHT in these tissues by the method of Anglin, Mahon and Chapman, as the superheated steam entrains volailte reducing substances which make the specific analysis of the BHT impossible.

The application of these methods has enabled us to obtain the results compiled in Tables I to IV. For the pig, the limit values and the individual values are shown. For the chicken, there are given the average contents and the contents corresponding to the fats from several animals, for the reason mentioned before.

Passage of the antioxidants into the tissues

The methods studied were applied to the different tissues and organs sampled under the above-described conditions.

Pig

Table I. Content of antioxidants in the tissues of the pig.

	Outer fatback %	Inner fatback %	Pork fat %	Muscle %	Liver %	Kidney %	Blood %
Propyl gallate	0	0	0	0	0	0	0
Dodecyl gallate	0	0	0	0	0	0	0
BHA	0	0	0	0	0	0	0
NDGA	0	0	0	0	0	0	0
BHT	0	0	0	0	0	0	0

Table I. continued.

DBPC	0	0	0	0	0	0	0
DPPD	0 to 0.0025	0 to 0.0024	0.0012 to 0.0024	0	0	0 to 0.006	0

The results of Table I show that the anitoxidants that can be determined under good conditions do not accumulate in adipose tissue, or in muscle, or in the liver or in the kidney, or in the blood. The gallic acid was also tested, as an experiment carried out in vitro had shown that the gallates are rapidly hydrolyzed by the intestinal content of the pig. For example, after incubation in this medium, it is possible to find the BHA quantitatively, whereas the gallates disappear completely. However, the qualitative tests for gallic acid ($FeCl_3$) applied to the fatty tissues and to the organs were always negative.

One antioxidant forms an exception to this rule - diphenylparaphenylendiamine (DPPD).

The levels of DPPD found in the fatback and in the porkfat are low in absolute value: maximum 0.0025%, or 25 mg per kg of fat. It should be noted that this low concentration is, however, sufficient to impart a faint pink color to the fat, in certain cases. We shall see, besides, that this concentration is also sufficient to retard its oxidation considerably.

Table II. DPPD content in various tissues of the pig. Individual values.

Pig	Outer fatback %	Inner fatback %	Pork-fat %	Liver %	Muscle %	Kidney %	Blood %
A	0.0022	0.0024	0.0024	0	0	0.0006	0
B	0	0.0014	0.0016	0	0	0.0006	0
C	0.0025	0.0024	0.0023	0	0	0	0
D	0.00094	0.00034	0.0012	0	0	0.0005	0
T	0	0	0.0023	0	0	0.0005	0
U	0.0022	0.0015	0.0024	0	0	0.0006	o

There are individual variations in the quantity of antioxidants contained in the fat (Table II). Among the other tissues and organs, the kidney is able to retain DPPD; the concentration found is of the order of 0.0005%, while neither the muscle, nor the liver, nor the blood contain measurable quantities of this antioxidant.

Chicken

The study of chicken tissue, summarized in Tables III and IV, shows that two antioxidants can accumulate in the fats of these birds - DPPD and BHT.

Table III. Average contents of antioxidants in chicken tissue.

	Fat %	Muscle %	Liver %
Propyl gallate	0	0	0
Dodecyl gallate	0	0	0
BHA	0	0	0
NDGA	0	0	0
BHT	0.006	0	0
DPPD	0.0007	0	0

Table IV. DPPD content of the various tissues of chicken.

	Fat %	Muscle %	Liver %
Group 9 (3 chicken)	0.001	0	0
Group 10 (3 chicken)	0.0005	0	0
Group 11 (3 chicken)	0.0007	0	0
Group 12 (4 chicken)	0.0005	0	0

However, the percentage of DPPD found in the chicken (0.0007%) is lower than that found in the pig. The concentrations are two to three times lower in the first case than in the second. These results were confirmed by the fact that the fats of the treated birds are not stabilized, while the induction period is substantially increased in the case of the pig.

On the other hand, the BHT (or DBPC), which it had not been possible to

into evidence in the pig fats, here exists at an easily determinable concentration (0.003%). BHT or related substances are indeed involved, because the fat of control animals did not give the color reaction of BHT. And, as in the case of DPPD in the pig, these analyses were confirmed by the study of the stability of the reserve fats. The induction period of the fats of the animals that had received BHT was substantially increased.

Influence of the ingestion of antioxidants on the stability of the fats to oxidation

The stability of the fats to oxidation is a function of two main factors which act in opposite direction - the proportion of unsaturated fatty acids and the proportion of antioxidants.

As to the first of these factors, we determined the iodine number on each sample. This gives, in first approximation, a measure of the quantity of unsaturated fatty acids present in the fat.

The average iodine numbers of the pig fats studied are summarized in Table V.

Table V. Iodine number of the adipose tissues of the pig.

	Outer fatback	Inner fatback	Pork fat
Control	62.3	55.5	48.4
BHT (1)	62.5	56.9	47.5
BHT (2)	64.2	58.7	49.4
BHA	62.7	56.6	49.6
Propyl gallate	61.4	55.5	49.3
Dodecyl gallate	65.2	61.1	51.8
NDGA	62.8	57.0	49.2
DPPD	64.7	57.3	47.7

The numbers are not notably changed in most of the lots. Therefore, the addition of antioxidants to the rations does not threaten to change the melting point of the fats considerably. However, there is a significant difference be-

tween the average iodine number of the control lot and that of the DPPD lot. This would amount to a "saving" of the unsaturated fatty acids in the animals having ingested antioxidant. This observation has been made before by Bratzler et al. (1950) who studied the composition of the fats of pigs. In the animals receiving tocopherols, there was accumulation of oleic acid at the expense of the saturated fatty acids. However, Garton et al. (1958) did not confirm these results, and Johnson, O'Halloran and Hegwill (1958) found an influence variable with the proportion of BHA contained in the ration.

For the antioxidants, we made a direct analysis, the results of which are shown in figures 1 to 4. We also used an indirect method which consists in studying the stability of the fats toward an artificially accelerated oxidation. Thus the kinetics of the evolution of the peroxide level of the fats is investigated. These samples, taken from animals which had received antioxidants, are compared on the one hand with the fats of control animals (without antioxidants) and, on the other, with samples of fat of control pigs to which known quantities of each of the antioxidants had been added. By comparing the duration of the induction periods, it is thus possible to determine the order of magnitude of the content of antioxidants in the fat tissue.

Pig

Figure 1 shows the evolution of the peroxide number of the fats maintained at 60°C. We calculated the average of the durations (in hours) necessary to attain peroxide numbers equal to 5, 10 and 20. This curve relates to the inner fatback.

Besides, figures 2 and 3 indicate the evolution of control fats enriched with given quantities of antioxidants - 0.001% and 0.001%.

Figure 1 (1) shows very clearly that the DPPD ingested by the animal considerably stabilizes the reserve fats toward oxidation. Besides, figure 2 shows that the addition of 0.001% of DPPD permits a peroxide number of 10 to be attained in about 200 hours; the value 20 is attained in 300 hours. This is the order of magnitude of the values shown in figure 1. It may be concluded there-

from that the order of magnitude of the DPPD concentration is 0.001%, while direct analysis leads to a level between 0.0012 and 0.0025%. The study of the kinetics of the peroxide level thus confirms the result of the direct analysis.

(p. 203) Fig. 1. Evolution of the peroxide number of samples of inner fatback of pig (animals having ingested the different antioxidants).

Fig. 2. Evolution of the peroxide number of inner fatback of pig added with 0.001% of DPPD or BHT.

Fig. 3. Evolution of the peroxide number of inner fatback of pig added with 0.0001% of DPPD or BHT.

The inner fatbacks of the lot having received the BHT seem slightly stabilized in relation to the other lots. The study of figure 2 also shows that BHT added to the fats at the dose of 0.001% leads to the appearance of a peroxide number equal to 20 after about 160 hours. However, the comparison of figures 1 and 3 permits us to conclude that the amount of antioxidants possibly stored in the fat would be less than 0.001%, and probably close to 0.0001% (index 20 after 80 hours). A slight passage of BHT may be presumed, therefore, but in too small a quantity to be brought into evidence by means of chemical analysis.

As to the other antioxidants, it is impossible to bring into evidence a significant stabilization of the fats. While propyl gallate increases the induction period, it is impossible to bring it into evidence by quantitative analysis. This may be due to the sensitivity of the analysis, or else to an indirect effect; in fact, synthetic antioxidant added to the diet may cause a "saving" of natural antioxidants such as the tochopherols. In most cases, however, the direct analysis in the tissue permits us to discard this hypothesis. In the case of propyl gallate, there may be involved the presence of a product of transformation of this bubstance, different from gallic acid, since the reaction with $FeCl_3$ was negative.

Chicken

The iodine number of the chicken fats was substantially higher (67 to 72) than that of the pig fats. Besides, the proportion of the DPPD is 3 to 4 times lower than that which corresponds to the pig fats. These two characteristics, however, modify the efficiency of the antioxidant. In fact, the fats of the chickens of the DPPD lot are practically not stabilized. On the contrary, the BHT, which we assayed at 0.006%, stabilizes the chicken fats considerably (fig. 4). The two methods, direct and indirect, checked against each other, thus give comparable information.

(p. 205) Fig. 4. Evolution of the peroxide number of samples of chicken fat (animals having ingested the different antioxidants).

Discussion

The results obtained in this research thus demonstrate the passage of certain antioxidants into the animal products. This passage relates mainly to the fat tissue. In the pig, only DPPD is capable of storage therein. Also in the chicken, DPPD is able to cross the intestinal barrier and to accumulate in the fats. The same is true of the BHT, but the concentration of this antioxidant attains much higher values than those for DPPD. The levels found are, however, low in absolute value: 0.005% maximum.

It should also be emphasized that the amount of 0.1 or 0.125% of antioxidant incorporated in the experimental diets is ten times higher than that recommended for stabilizing the fats. However, the stabilization of certain foods (especially animal flours) may require high concentrations of antioxidants. Likewise, the rate of incorporation of antioxidants in poultry feed to prevent encephalomalacia may be relatively high. Yet, the concentrations we have used in these diets represent a maximum which would not be reached in the practice. Consequently, it may be presumed that the amounts found in the tissues would be notably lower than those we have observed.

Nevertheless, the use of antioxidants in animal nutrition poses a twofold problem. From the view point of the technology of animal products, the accum-

ulation of certain antioxidants in the tissues, and notably in the fat tissues, constitutes a favorable factor, as it has as corollary a better resistance to the oxidation of these fats. The hygienist, however, must demand that the antioxidant present in the tissues should pose no problem to the consumer of pork or chicken meat. We now know the toxicity of DPPD (Ames et al., 1956); Oser and Oser (1956) have shown that this antioxidant brings about an increase in the gestation period in the rat, from which a high mortality among the dams and the young results. It seems to be well established, on the other hand, that BHT is a substance of very low toxidity (Deichman et al., 1955).

In view of the stability of both DPPD and BHT toward chemical agents, it is very likely that the molecule of these substances has not been modified during intestinal absorption and transport.

Moreover, the good agreement between the direct analysis of the antioxidants and the induction period of the fats supports this opinion. However, the problem of the transformation products of the ingested products should not be minimized. Johnson, O'Hallorgan and Hegwill (1959) have stressed the difficulties involved in identifying these products.

(For Summary in English see p. 206)

Translated by Carl Demrick Associates, Inc./LH/db

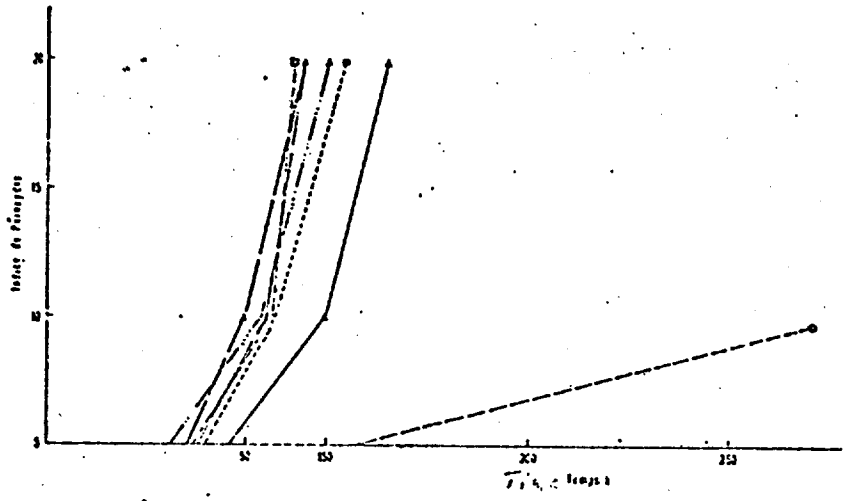

FIG. 1. — Évolution de l'indice de péroxydes d'échantillons de lard interne de porc (animaux ayant ingéré les différents antioxygènes).

□— — — — — témoin
△—···—···— NDGA
△—— —— —— BHA
△—·—·—· Gallate de dodecyle
○····· Gallate de opylpre
△———— BHT
○— — — — DPPD

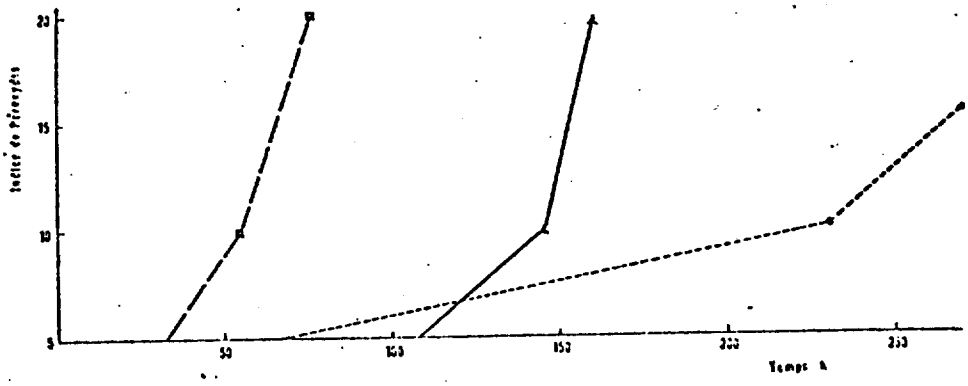

FIG. 2. — Évolution de l'indice de péroxydes de lard interne de porc additionné de 0,001 p. 100 de DPPD ou de BHT.

□— — — — témoin
△———— BHT
○- - - - - - DPPD

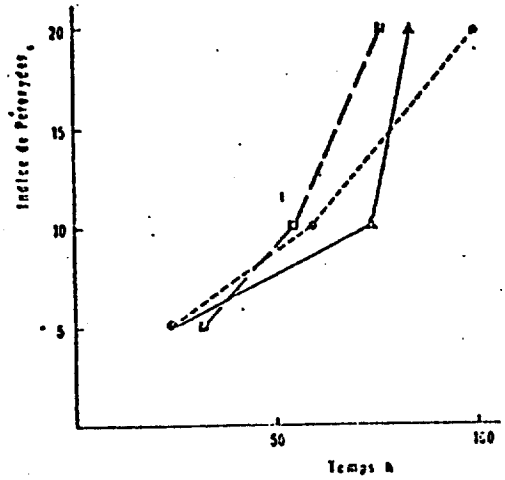

FIG. 3 — Évolution de l'indice de péroxydes de lard interne de porc additionné de 0,0001 p.100 de DPPD ou de BHT.

□——— témion
△——— BHT
○------ DPPD

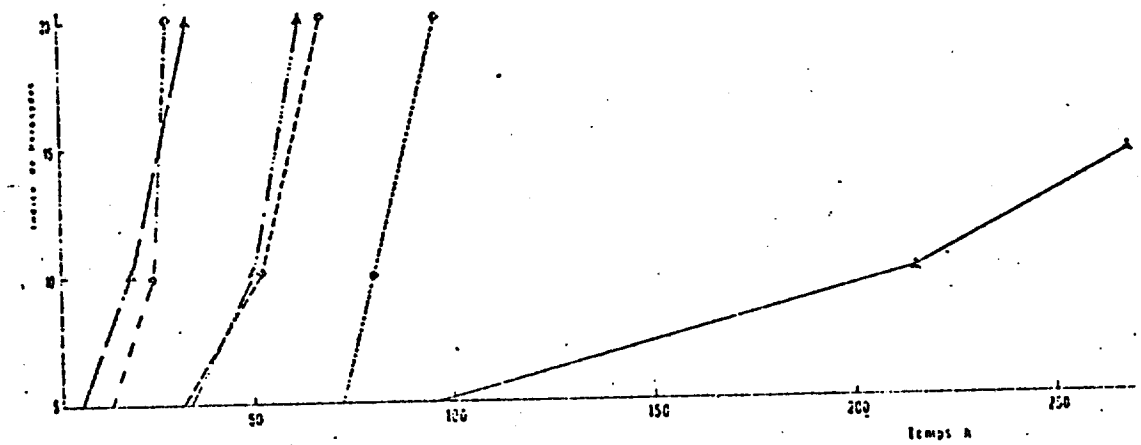

FIG. 4. — Évolution de l'indice de péroxydes d'échantillons de graisse de poulets (animaux ayant ingéré les différents antioxygènes).

△-·-·-·- NDGA
△— — — BHA
○-·-·-·- Gallate de dodecyle
△..... Gallate de propyle
○——— BHT
○------ DPPD

ANN. ZOOTECH.

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INFLUENCE DE L'INGESTION D'ANTIOXYGÈNES SUR LA COMPOSITION DE CERTAINS TISSUS ET SUR LA STABILITÉ DES GRAISSES DE RÉSERVE DU PORC ET DU POULET

PAR

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SOMMAIRE

L'influence de l'ingestion de divers antioxygènes sur leur accumulation éventuelle dans le muscle, les graisses de réserve (dorsale et périnéphrétique), le foie et le rein, ainsi que sur la résistance à l'oxydation des graisses de réserve, a été étudiée chez le porc et le poulet.

Les animaux recevaient dans leur ration soit 0,125 p. 100 de diphényl-p. phénylènediamine (DPPD), soit 0,1 p. 100 de butylhydroxyanisol (BHA), de butylhydroxytoluène (BHT), de gallate de dodécyle, de gallate de propyle, ou d'acide nordihydroguaïarétique (NDGA).

Chez le porc, seul le DPPD est décelable dans les graisses de réserve (taux maximum trouvé : 0,0025 p. 100), et dans le rein (maximum 0,0060 p. 100). Chez le poulet, le DPPD s'accumule également dans les graisses de réserve, mais la concentration est plus faible (0,0007 p. 100) que dans la graisse de porc. En revanche, le BHT est stocké dans la graisse de poulet au taux de 0,006 p. 100.

L'étude de la stabilité des graisses de réserve des animaux témoins et des animaux traités confirme le résultat du dosage direct des antioxygènes. En effet, les graisses provenant des porcs ayant ingéré du DPPD et celles des poulets ayant reçu du BHT présentent une période d'induction beaucoup plus longue que celle des graisses d'animaux témoins.

Les conséquences technologiques et hygiéniques de ces observations sont discutées.

Certains antioxygènes naturels tels que les tocophérols, contenus normalement dans le régime alimentaire des animaux, sont capables d'accroître la résistance à l'oxydation des graisses de réserve. Ce fait a été démontré chez le rat (BARNES et al. 1943 ; LUNDBERG et al. 1944 ; HANSON et al. 1944 ; BURR, LUNDBERG et CHIPAULT 1946 ; LUNDBERG et al. 1947), le lapin (MAJOR et WATTS, 1948), le porc (CARPENTER et LUNDBERG 1949 ; CHIPAULT, LUNDBERG et BURR, 1945 ; WATTS, CUNHA et MAJOR, 1946), le poulet (MECCHI et al. 1956, a b ; HOOD, WHEELER et McGLAMERY, 1950), le dindon (MECCHI, POOL et KLOSE, 1953 ; CRIDDLE et MORGAN, 1951).

Les recherches portant sur d'autres antioxygènes et notamment sur les produits de synthèse sont peu nombreuses. Lorsque le présent travail a été entrepris, en 1957, l'un des rares travaux publiés portait sur le métabolisme de la diphényl-p.-phénylènediamine chez le poulet (PUDELKIEWICZ et al. 1956). Un peu plus tard une courte étude sur le porc a été publiée (LYNCH, ANDERSON et LEWIS, 1958). Antérieurement BARNES et al. (1943) avaient montré que l'hydroquinone ingérée, loin de stabiliser les graisses de réserve du rat, provoquait au contraire une accélération de l'oxydation. Or, l'utilisation des antioxygènes en alimentation animale est le corollaire obligatoire de l'incorporation des graisses aux aliments. Ces substances peuvent être également utilisées pour la stabilisation des aliments contenant des graisses oxydables tels que les farines de poisson. On sait en effet, que l'oxydation des graisses alimentaires risque de provoquer, chez les animaux qui les ingèrent, des accidents nutritionnels dont l'origine résulte directement ou indirectement de la présence des produits d'oxydation. Ainsi, afin de prévenir certains de ces accidents tels que l'encéphalomalacie, on incorpore certains antioxygènes à la ration alimentaire des animaux.

Le présent travail a pour objet d'étudier chez le porc et le poulet quelles sont les conséquences de l'ingestion de divers antioxygènes sur la composition des tissus animaux consommés par l'homme.

MATÉRIEL et MÉTHODES

Antioxygènes utilisés — animaux

Les antioxygènes suivants ont été étudiés : gallate de propyle ; gallate de dodécyle ; butylhydroxytoluène (BHT) (ou dibutylparacrésol : DBPC) ; acide nordihydroguaiarétique (NDGA) ; butylhydroxyanisole (BHA) ; diphénylparaphénylènediamine (DPPD). La dose incorporée aux aliments était de 0,1 p. 100 pour tous les produits, sauf pour le DPPD qui était incorporé au taux de 0,125 p. 100. Ce taux correspond à dix fois

celui qui est généralement recommandé pour stabiliser les graisses alimentaires.

Pour les pores, la durée d'administration du régime enrichi d'antioxygènes était de quatre mois environ (poids de 50 à 100 kg). Chacun des lots, dont un lot témoin, comprenait 6 animaux. Pour les poulets, l'expérience avait une durée de huit semaines. Chacun des lots comprenait 15 oiseaux.

Organes et tissus étudiés

Chez le porc, le lard dorsal était prélevé dès l'abattage. Les deux couches qui constituent ce tissu étaient séparées et analysées séparément. En outre, on prélevait les graisses périnéphrétiques (panne). Ces tissus gras étaient fondus sous atmosphère de CO_2, filtrés et centrifugés. Les déterminations suivantes étaient effectuées sur ces graisses : taux d'antioxygènes, indice d'iode, indice de péroxydes, test de stabilité. Ce dernier test était effectué à l'étuve 60°C, éclairée, selon une méthode recommandée par DESNUELLE et par WOLFF. Le muscle semi-tendineux, le foie et le rein faisaient également l'objet de prélèvements à des fins d'analyses. Chez le poulet, les graisses de réserve étaient souvent absentes, bien que nous ayons conduit les animaux jusqu'à 16 semaines, afin d'obtenir un engraissement suffisant. Nous n'avons donc pu étudier les réserves individuelles. Pour obtenir une quantité suffisante de graisses, nous avons dû réunir les prélèvements correspondant à deux ou trois animaux. Pour le tissu musculaire, les muscles pectoraux et les muscles de la patte ont été prélevés.

Dosage

Graisses

Les indices d'iode étaient déterminés selon la méthode de WIJS et les indices de péroxydes (Lea) par iodométrie.

Antioxygènes

La méthode de KAHAN (1954) à l'α-α' dipyridyl a été utilisée pour doser le NDGA et le BHA. Elle a été également appliquée au dosage des gallates. En effet, la ration de chacun des lots ne contenait qu'un seul antioxygène et, en conséquence, le manque de spécificité de la réaction, qui est un obstacle lorsque l'on a affaire à un mélange, ne présentait pas d'inconvénient. Il est nécessaire de tracer une courbe d'étalonnage pour chacune des séries de dosages mettant en œuvre cette réaction, quel que soit l'antioxygène dosé. Les lectures étaient effectuées au spec-

trophotomètre à 515 mμ ; le temps de réaction était de 30 mn pour le BHA et les gallates, et de 3 mn pour le NDGA. La modification introduite par ANGLIN, MAHON et CHAPMAN était appliquée pour le dosage du BHT (addition de n-butanol).

Pour le DPPD on préparait le dérivé nitré qui est dosé par colorimétrie (BUNNEL, 1954). Dans le cas du dosage de cet antioxygène dans les graisses, nous avons dû procéder à une saponification préalable, après avoir vérifié que celle-ci ne modifiait pas la validité du dosage (5 g de graisse, 2 g de KOH, 50 ml d'alcool, pendant une demi-heure à l'ébullition).

L'extraction des antioxygènes à partir des tissus et organes pose certains problèmes. En particulier, le BHT doit être entraîné par la vapeur surchauffée, selon la méthode de ANGLIN, MAHON et CHAPMAN (1956). Nous avons cependant trouvé que la température de distillation proposée par ces auteurs ne convenait pas ; elle doit être portée à 180°C afin d'obtenir une récupération quantitative de l'antioxygène.

Dans le cas des tissus gras, ceux-ci sont dissous dans l'éther de pétrole. L'antioxygène (BHA, NDGA, gallates) est réextrait par l'alcool 72 p. 100. Pour le muscle, l'extraction du BHA et des gallates s'effectue à l'aide d'éther de pétrole, au blendor, selon la méthode de HANLEY (1954). Par ailleurs, la présence de gallates à des concentrations extrêmement faibles peut être détectée par addition de quelques gouttes d'ammoniaque concentré (WENGER, 1954).

RÉSULTATS

Validité des méthodes de dosage

Des tests de surcharge ont été effectués avec les différents antioxygènes étudiés, afin de déterminer la limite inférieure de sensibilité, ainsi que la validité du dosage. Ces surcharges ont été effectuées sur le tissu gras, sur le muscle, sur le foie et sur le rein. Les concentrations étudiées variaient de 0,001 p. 100 à 0,1 p. 100.

a) GRAISSE.

Les taux de récupération atteignent pour plusieurs antioxygènes des valeurs de l'ordre de 95 p. 100, même pour des concentrations aussi faibles que 0,001 p. 100.

Toutefois, la récupération est plus faible (90 p. 100 environ) pour ce qui concerne le gallate de propyle et le BHT. Dans ce dernier cas, la succession des opérations de dosage, qui comporte notamment un entraînement à la vapeur surchauffée, est vraisemblablement responsable de ce taux relativement faible. En aucun cas, il n'a été possible de

confirmer les résultats de ANGLIN, MAHON et CHAPMAN. En appliquant strictement la méthode proposée par ces auteurs, sans modifier la température de distillation, les taux de récupération ne dépassaient pas 50 à 60 p. 100.

b) MUSCLE ET ORGANES.

Les récupérations des surcharges sont également de l'ordre de 95 p. 100 pour ce qui concerne le muscle. Pour le rein et le foie, certaines difficultés se présentent pour le DPPD et le gallate de propyle. Enfin, nous avons dû renoncer à doser le BHT dans ces tissus au moyen de la méthode de ANGLIN, MAHON et CHAPMAN. En effet, la vapeur surchauffée entraîne des substances volatiles réductrices qui rendent impossible le dosage spécifique du BHT.

L'application de ces méthodes nous a permis d'obtenir les résultats réunis dans les tableaux I à IV. On a indiqué , pour le porc, les valeurs extrêmes et les valeurs individuelles. Pour le poulet, on trouve les teneurs moyennes et les teneurs correspondant aux graisses provenant de plusieurs animaux, pour la raison déjà mentionnée.

Passage des antioxygènes dans les tissus

Les méthodes étudiées ont été appliquées aux différents tissus et organes prélevés dans les conditions décrites ci-dessus.

Porc

TABLEAU I

Teneur en antioxygènes des tissus du porc

	Lard externe	Lard interne	Panne	Muscle	Foie	Rein	Sang
	p. 100	p. 100	p. 100	p. 100	p. 100	p. 100	p. 100
Gallate de propyle	0	0	0	0	0	0	0
Gallate de dodécyle	0	0	0	0	0	0	0
BHA	0	0	0	0	0	0	0
NDGA	0	0	0	0	0	0	0
BHT	0	0	0	0	0	0	0
DHPC	0	0	0	0	0	0	0
DPPD	0 à 0,0025	0 à 0,0024	0,0012 à 0,0024	0	0	0 à 0,0006	0

Les résultats du tableau I montrent que les antioxygènes qu'il est possible de doser dans de bonnes conditions ne s'accumulent pas dans le tissu adipeux, ni dans le muscle, ni dans le foie, ni dans le rein, ni dans le sang. L'acide gallique a été également recherché, car un essai effectué in-vitro a montré que les gallates sont rapidement hydrolysés par le contenu intestinal du porc. Par exemple, après incubation dans ce milieu, il est possible de retrouver quantitativement le BHA, alors que les gal-

lates disparaissent complètement. Or, les tests qualitatifs de l'acide gallique ($Fe\ Cl_3$) appliqués aux tissus gras et aux organes ont toujours été négatifs.

Un seul antioxygène, toutefois, fait exception à cette règle : il s'agit de la diphénylparaphénylènediamine (DPPD).

Les taux de DPPD trouvés dans le lard et dans la panne sont toutefois faibles en valeur absolue : 0,0025 p. 100 au maximum, soit 25 mg par kg de graisse. Notons que cette faible concentration suffit néanmoins à communiquer, dans certains cas, à la graisse une faible coloration rose. Nous verrons par ailleurs que cette concentration suffit également à en retarder considérablement l'oxydation.

TABLEAU II

Teneur de divers tissus du porc en DPPD
Valeurs individuelles

Porc	Lard externe	Lard interne	Panne	Foie	Muscle	Rein	Sang
	p. 100	p. 100	p. 100	p. 100	p. 100	p. 100	p. 100
A	0,0022	0,0024	0,0024	0	0	0,0006	0
B	0	0,0014	0,0016	0	0	0,0006	0
C	0,0025	0,0024	0,0023	0	0	0	0
D	0,00094	0,00094	0,0012	0	0	0,0005	0
T	0	0	0,0023	0	0	0,0005	0
U	0,0022	0,0015	0,0024	0	0	0,0006	0

Il existe des variations individuelles dans la quantité d'antioxygènes contenue dans la graisse (tableau II). Enfin, parmi les autres tissus et organes, le rein est capable de retenir du DPPD ; la concentration trouvée est de l'ordre de 0,0005 p. 100, alors que ni le muscle, ni le foie, ni le sang ne contiennent de quantités dosables de cet antioxygène.

Poulet.

L'étude des tissus du poulet, résumée dans les tableaux III et IV

TABLEAU III

Teneurs moyennes en antioxygènes des tissus de poulets

	Graisse	Muscle	Foie
	p. 100	p. 100	p. 100
Gallate de propyle	0	0	0
Gallate de dodécyle	0	0	0
BHA	0	0	0
NDGA	0	0	0
BHT	0,006	0	0
DPPD	0,0007	0	0

TABLEAU IV

Teneur des divers tissus du poulet en DPPD.

	Graisse	Muscle	Foie
	p. 100	p. 100	p. 100
Groupe 9 (3 poulets)	0,001	0	0
Groupe 10 (3 poulets)	0,0005	0	0
Groupe 11 (3 poulets)	0,0007	0	0
Groupe 12 (4 poulets)	0,0005	0	0

montre que deux antioxygènes sont capables de s'accumuler dans les graisses de ces oiseaux : le DPPD et le BHT.

Toutefois, le taux de DPPD retrouvé chez le poulet (0,0007 p. 100) est inférieur à celui qui a été trouvé chez le porc. Les concentrations sont deux à trois fois plus faibles dans le premier cas que dans le second. Ces résultats ont été confirmés par le fait que les graisses des oiseaux traités ne sont pas stabilisées, alors que la période d'induction est notablement accrue dans le cas du porc.

En revanche, le BHT (ou DBPC) qu'il n'avait pas été possible de mettre en évidence dans les graisses du porc, existe ici à une concentration aisément dosable (0,006 p. 100). Il s'agit bien de BHT ou de corps voisins, car la graisse d'animaux témoins ne donnait pas la réaction colorée du BHT. Enfin, comme dans le cas de DPPD chez le porc, ces dosages ont été confirmés par l'étude de la stabilité des graisses de réserve. La période d'induction des graisses des animaux ayant reçu du BHT était notablement augmentée.

Influence de l'ingestion d'antioxygènes sur la stabilité des graisses vis-à-vis de l'oxydation

La stabilité des graisses vis-à-vis de l'oxydation est fonction de deux facteurs principaux qui agissent en sens opposé : d'une part le taux d'acides gras insaturés et, d'autre part le taux d'antioxygènes.

Pour ce qui concerne le premier de ces facteurs, nous avons déterminé sur chacun des échantillons l'indice d'iode qui, en première approximation, donne une mesure de la quantité d'acides gras insaturés présents dans la graisse.

Les indices d'iode moyens des graisses de porc étudiées sont résumés dans le tableau V.

Les indices ne sont pas notablement modifiés dans la plupart des lots. En conséquence, l'addition d'antioxygènes aux rations ne risque donc pas de modifier considérablement le point de fusion des graisses.

TABLEAU V

Indice d'iode des tissus adipeux du porc

	Lard externe	Lard interne	Panne
Témoin	62,3	55,5	48,4
BHT (1)	62,5	56,9	47,5
BHT (2)	64,2	58,7	49,4
BHA	62,7	56,6	49,6
Gallate de Propyle	61,4	55,5	49,3
Gallate de Dodécyle	65,2	61,1	51,8
NDGA	62,8	57,0	49,2
DPPD	64,7	57,3	47,7

Toutefois, il existe une différence significative entre l'indice d'iode moyen du lot témoin et celui du lot DPPD. Il y aurait donc « épargne » des acides gras insaturés chez les animaux ayant ingéré l'antioxygène. Cette observation a déjà été faite par BRATZLER et al. (1950) qui étudiaient la composition des graisses de porcs. Chez les animaux recevant des tocophérols, il y avait accumulation d'acide oléique aux dépens des acides gras saturés. Toutefois, GARTON et al. (1958) n'ont pas confirmé ces résultats, et JOHNSON, O'HALLORAN et HEGWILL (1958) ont constaté une influence variable selon le taux de BHA contenu dans la ration.

Pour les antioxygènes, nous avons effectué le dosage direct dont les résultats sont mentionnés dans les tableaux I à IV. En outre, nous avons utilisé une méthode indirecte qui consiste à étudier la stabilité des graisses vis-à-vis d'une oxydation accélérée artificiellement. On étudie ainsi la cinétique de l'évolution du taux des péroxydes des graisses Ces échantillons qui ont été prélevés sur des animaux ayant reçu des antioxygènes sont comparés d'une part aux graisses d'animaux témoins (sans antioxygènes) et, d'autre part à des échantillons de graisse de porcs témoins, additionnés de quantités connues de chacun des antioxygènes. Par comparaison de la durée des périodes d'induction, il est ainsi possible de déterminer l'ordre de grandeur de la teneur en antioxygènes du tissu gras.

Porc.

La figure 1 montre l'évolution de l'indice de péroxydes des graisses maintenues à 60°C. Nous avons calculé la moyenne des durées (en heures) nécessaires pour atteindre des indices de péroxydes égaux à 5, 10 et 20. Cette courbe concerne le lard interne.

Par ailleurs, les figures 2 et 3 indiquent l'évolution de graisses témoins enrichies de quantités données d'antioxygènes : 0,001 p. 100 et 0,0001 p. 100.

La figure 1 (1) montre très clairement que le DPPD ingéré par l'animal stabilise considérablement les graisses de réserve vis-à-vis de

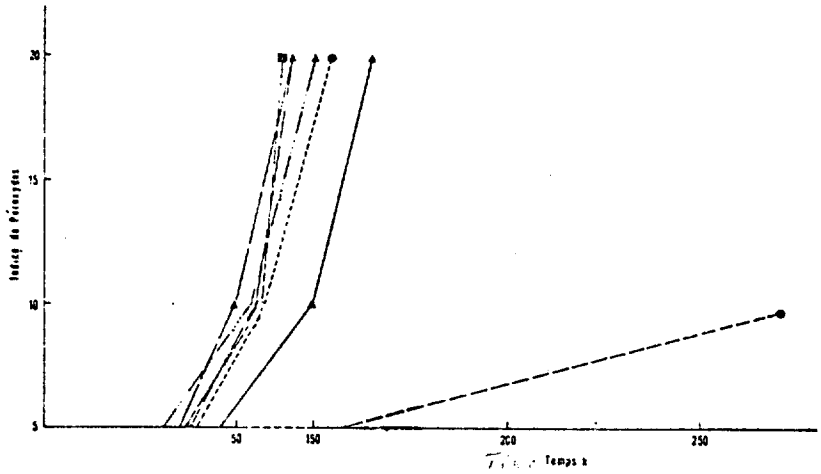

FIG. 1. — Évolution de l'indice de péroxydes d'échantillons de lard interne de porc (animaux ayant ingéré les différents antioxygènes).

□— — — — —	témoin	△—·—·—·	Gallate de dodecyle
△—···—···—	NDGA	○·····	Gallate de opylpre
▲———	BHA	△———	BHT
		○— — — —	DPPD

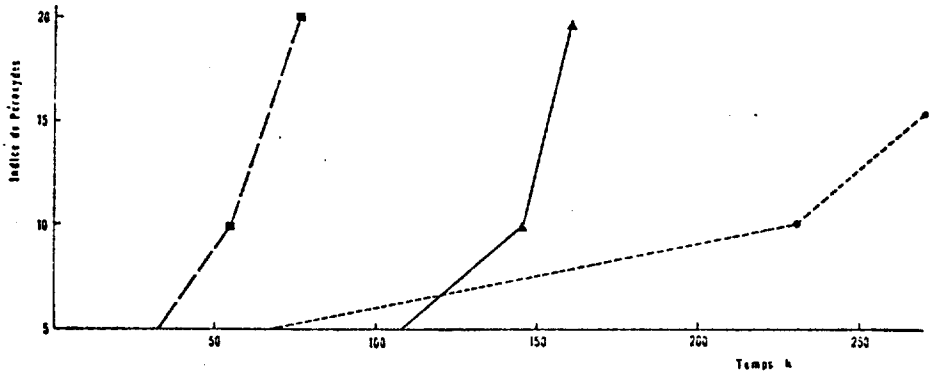

FIG. 2. — Évolution de l'indice de péroxydes de lard interne de porc additionné de 0,001 p. 100 de DPPD ou de BHT.

□— — — —	témoin
△———	BHT
○- - - - - -	DPPD

l'oxydation. Par ailleurs, la figure 2 montre que l'addition de 0,001 p. 100 de DPPD permet d'atteindre un indice de péroxydes égal à 10 en 200 heures environ ; la valeur 20 est atteinte en 300 heures. Or, c'est l'ordre de grandeur des valeurs relevées sur la figure 1. On peut en conclure que l'ordre de grandeur de la concentration en DPPD est 0,001 p.100, alors que le dosage direct aboutit à un taux compris entre 0,0012 et 0,0025 p. 100. L'étude de la cinétique du taux de péroxydes confirme donc le résultat du dosage direct.

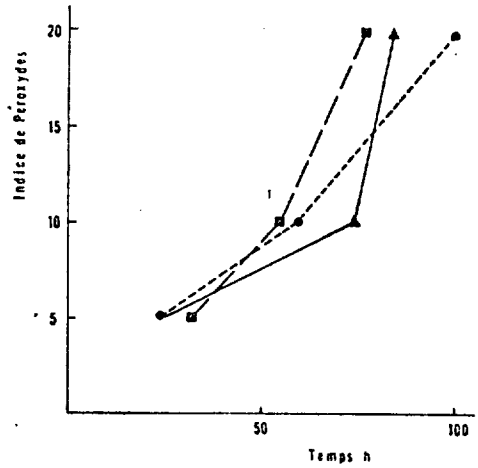

FIG. 3 — Évolution de l'indice de péroxydes de lard interne de porc additionné de 0,0001 p. 100 de DPPD ou de BHT.

■——— témion
▲——— BHT
○------ DPPD

Les lards internes du lot ayant reçu le BHT semblent légèrement stabilisés par rapport aux autres lots. L'étude de la figure 2 montre également que le BHT ajouté aux graisses à la dose de 0,001 p. 100 conduit à l'apparition d'un indice de péroxydes égal à 20 après environ 160 heures. Toutefois, la comparaison des figures 1 et 3 permet de conclure que le taux d'antioxygènes éventuellement stocké dans la graisse serait inférieur à 0,001 p. 100, et vraisemblablement voisin de 0,0001 p. 100 (indice 20 après 80 heures). En conséquence, on peut présumer un léger passage de BHT, mais en quantité trop faible pour qu'il puisse être mis en évidence au moyen d'un dosage chimique.

Pour les autres antioxygènes, il est impossible de mettre en évidence une stabilisation significative des graisses. Le gallate de propyle, toutefois, accroît la période d'induction, bien qu'il soit impossible de le mettre en évidence par dosage. Ceci peut être dû à la sensibilité du dosage, ou bien à un effet indirect ; en effet, l'antioxygène de synthèse ajouté au régime pourrait provoquer une « épargne » d'antioxygènes naturels tels

que les tocophérols. Toutefois, dans la plupart des cas, le dosage direct dans le tissu permet d'écarter cette hypothèse. Dans le cas du gallate de propyle, il pourrait s'agir de la présence d'un produit de transformation de cette substance, différent de l'acide gallique, puisque la réaction avec $FeCl_3$ était négative.

Poulet.

L'indice d'iode des graisses de poulet est notablement plus élevé (67 à 72) que celui des graisses de porc. Par ailleurs, le taux du DPPD est 3 à 4 fois plus faible que celui qui correspond aux graisses de porc. Or, ces deux caractéristiques modifient l'efficacité de l'antioxygène. En effet, les graisses des poulets du lot DPPD ne sont pratiquement pas stabilisées. En revanche, le BHT, que nous avons par ailleurs dosé au taux de 0,000 p. 100, stabilise considérablement les graisses du poulet (fig. 4). Les deux méthodes, directe et indirecte, donnent ainsi par recoupement un renseignement comparable.

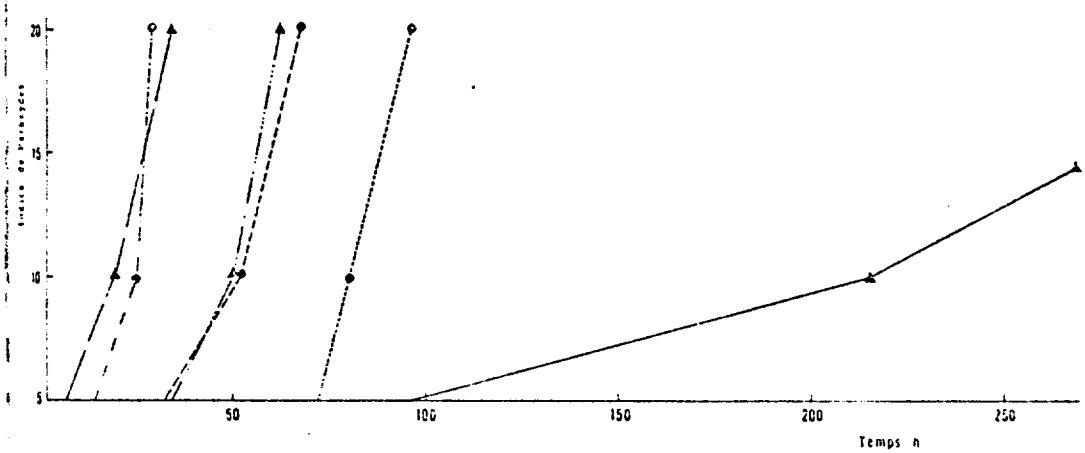

FIG. 4. — Évolution de l'indice de péroxydes d'échantillons de graisse de poulets (animaux ayant ingéré les différents antioxygènes).

▲—·—·—·· NDGA
▲— — — BHA
O—·—·—· Gallate de dodecyle
▲..... Gallate de propyle
O———— BHT
O- - - - - - DPPD

DISCUSSION

Les résultats obtenus au cours de ces recherches ont ainsi permis de mettre en évidence le passage de certains antioxygènes dans les productions animales. Ce passage concerne essentiellement le tissu gras dans lequel, chez le porc, le DPPD est seul pratiquement capable de se stocker. Chez le poulet, le DPPD est également capable de franchir la barrière intestinale et de s'accumuler dans les graisses. Il en est de même pour le BHT, mais la concentration de cet antioxygène atteint

que les tocophérols. Toutefois, dans la plupart des cas, le dosage direct dans le tissu permet d'écarter cette hypothèse. Dans le cas du gallate de propyle, il pourrait s'agir de la présence d'un produit de transformation de cette substance, différent de l'acide gallique, puisque la réaction avec $FeCl_3$ était négative.

Poulet.

L'indice d'iode des graisses de poulet est notablement plus élevé (97 à 72) que celui des graisses de porc. Par ailleurs, le taux du DPPD est 3 à 4 fois plus faible que celui qui correspond aux graisses de porc. Or, ces deux caractéristiques modifient l'efficacité de l'antioxygène. En effet, les graisses des poulets du lot DPPD ne sont pratiquement pas stabilisées. En revanche, le BHT, que nous avons par ailleurs dosé au taux de 0,006 p. 100, stabilise considérablement les graisses du poulet (fig. 4). Les deux méthodes, directe et indirecte, donnent ainsi par recoupement un renseignement comparable.

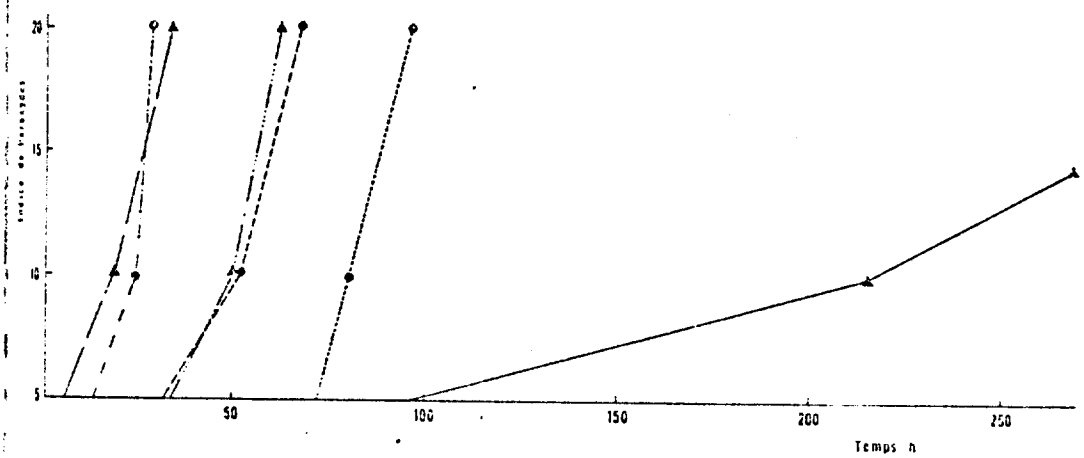

FIG. 4. — Évolution de l'indice de péroxydes d'échantillons de graisse de poulets (animaux ayant ingéré les différents antioxygènes).

NDGA — Gallate de propyle
BHA — BHT
Gallate de dodecyle — DPPD

DISCUSSION

Les résultats obtenus au cours de ces recherches ont ainsi permis de mettre en évidence le passage de certains antioxygènes dans les productions animales. Ce passage concerne essentiellement le tissu gras dans lequel, chez le porc, le DPPD est seul pratiquement capable de se stocker. Chez le poulet, le DPPD est également capable de franchir la barrière intestinale et de s'accumuler dans les graisses. Il en est de même pour le BHT, mais la concentration de cet antioxygène atteint

des valeurs très supérieures à celles qui correspondent au DPPD. Les taux trouvés sont toutefois faibles en valeur absolue : l'ordre de grandeur est au maximum de 0,005 p. 100.

Il convient également de souligner que le taux de 0,1 ou de 0,125 p. 100 d'antioxygène incorporé aux régimes expérimentaux est dix fois plus élevé que celui qui est recommandé pour stabiliser les graisses. Toutefois, la stabilisation de certains aliments (farines animales notamment) peut exiger des concentrations élevées d'antioxygènes. De même, le taux d'incorporation d'antioxygènes aux aliments destinés aux volailles, afin de prévenir l'encéphalomalacie peut être relativement élevé. Néanmoins, les concentrations que nous avons utilisées dans ces régimes constituent un maximum qui ne serait pas atteint dans la pratique. En conséquence, on peut présumer que les taux retrouvés dans les tissus seraient notablement plus faibles que ceux que nous avons observés.

Néanmoins, l'utilisation des antioxygènes dans l'alimentation des animaux pose un problème qui présente un double aspect. Du point de vue de la technologie des produits animaux, l'accumulation de certains antioxygènes dans les tissus, et notamment dans les tissus gras, constitue un facteur favorable puisqu'elle a pour corollaire une meilleure résistance à l'oxydation de ces graisses. Toutefois, pour l'hygiéniste, il est indispensable d'exiger que l'antioxygène présent dans les tissus ne présente aucun inconvénient pour le consommateur de viande de porc ou de poulet. Or, on connait maintenant la toxicité du DPPD (Ames et al., 1956) ; Oser et Oser (1956) ont montré que cet antioxygène provoque un accroissement de la durée de la gestation chez la rate, et qu'il en résulte une forte mortalité chez les mères et les jeunes. En revanche, il parait bien établi que le BHT est un corps très peu toxique (Deichman et al., 1955).

Compte tenu de la stabilité soit du DPPD, soit du BHT, vis-à-vis des agents chimiques, il y a de fortes présomptions pour que la molécule de ces corps n'ait pas été modifiée au cours de l'absorption intestinale, et du transport.

Par ailleurs, la bonne concordance qui existe entre le dosage direct des antioxygènes et la période d'induction des graisses permet de renforcer cette opinion. Toutefois, le problème des produits de transformation des produits ingérés ne doit pas être minimisé. Johnson, O'Halloran et Hegwil (1950) ont souligné les difficultés qui s'attachent à l'identification de ces produits.

SUMMARY

The influence of the ingestion of various antioxidants on their final accumulation in muscle, reserve fats (dorsal and perinephric), liver and kidney, and on the resistance to oxidation of the reserve fats, has been studied in the pig and the chicken.

The animals received in the ration either 0,125 p. 100 diphenyl-p. pheny-

lenediamine (DPPD) or 0,1 p. 100 butylated hydroxyanisole (B. H. A.), butylated hydroxytoluene (BHT), dodecyl gallate, propyl gallate, or nordihydroguaiaretic acid (NDGA).

The experiment lasted four months for the pig and eight weeks for the chicken. The BHA, NDGA and gallates were extracted with petroleum ether and reextracted with 72 p. 100 alcohol. They were determined by means of the $FeCl_3$-dipyridyl reaction (KAHAN, 1954). The BHT was distilled over with superheated steam, at 180°C (modification of the method of ANGLIN, MAHON, CHAPMAN, 1956) and also determined colorimetrically. The DPPD was extracted after saponification of the fatty tissue and its nitrated derivative determined by colorimetry. (BUNNEL, 1954).

In the pig, only DPPD is detectable in the reserve fats (maximum amount found : 0,0025 p. 100,. (Tables I and II), and in the kidney (maximum 0,0006 p. 100). In the chicken, DPPD also accumulates in the reserve fats, but the concentration is lower (0,0007 p. 100) than in the fat of the pig (Table III). On the other hand, BHT is stored in the fat of the chicken at a level of 0,006 p. 100.

The study of the stability of reserve fats (development of the peroxide index, in an oven regulated at 60°C and illuminated) of control and treated animals confirms the result of direct determination of the antioxidants. Indeed, the fats of pigs which had ingested DPPD (fig. 1) and those of chickens which had received BHT (fig. IV) show an induction period which is much longer than that of the fats of control animals. However, one observes induction periods of the same order of magnitude on the addition of known quantities of BHT or DPPD (0,001 and 0,0001 p. 100) to the fats of control pigs (fig. 2 and 3).

The technological and hygienic consequences of these observations are discussed.

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

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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-

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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.

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[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.

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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.

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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.

TABLE 3.

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	238	—	—
	D	10 p.c. lard	Male	23·3	266	—	—	—	—	23·1	272	12·6***	196***
			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.

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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.

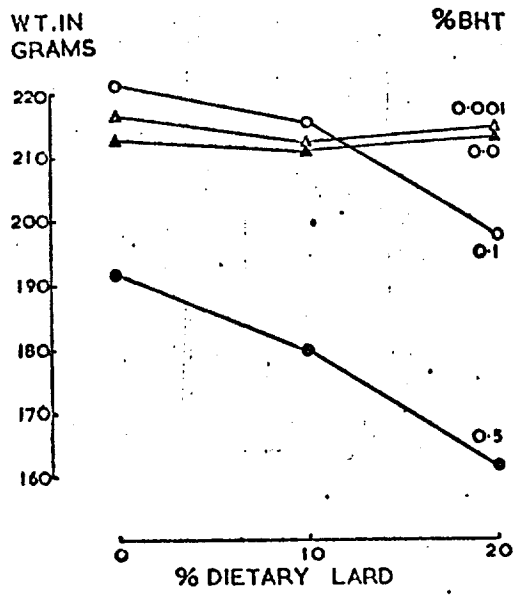

Fig. 1. The effect of a six-week dietary regimen of lard and BHT on the weight of pair-fed male rats.

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

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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 ($p < 0{\cdot}01$).

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-

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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

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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

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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 χ^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).

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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 dis-

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pensed 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 ($p < 0{\cdot}1$) 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

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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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THE EFFECT OF THE ANTIOXIDANTS, BUTYLATED HYDROXY ANISOLE, BUTYLATED HYDROXY TOLUENE AND PROPYL GALLATE ON GROWTH, LIVER AND SERUM LIPIDS AND SERUM SODIUM LEVELS OF THE RAT[1]

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SUMMARY.

Confirmation of our previous finding that there are greater hazards in the use of butylated hydroxy toluene (BHT) than in that of butylated hydroxy anisole (BHA) as antioxidants for use in human foodstuffs was obtained. BHA and propyl gallate (PG) met one of the requirements for safety as a food additive. A sex difference in the effect of BHT was noted; BHT increased the relative weight of the male adrenal and had a greater deleterious effect on the growth rate of the male than of the female rat. A straight line relationship existed between the dietary level of BHT and the serum cholesterol level. When fed at the level of 0·1 p.c. of the diet, BHA increased the level of total serum cholesterol but no further elevations occurred at higher dosages. There was a relatively greater increase in the amount of serum free cholesterol than of ester cholesterol when BHA or BHT were fed. PG had no effect on the serum cholesterol levels. BHT increased both free and esterified adrenal cholesterol, a greater increase being noted in the latter. Although BHA and BHT produced increases in the size of the male adrenal none of the marked histological changes recorded by other workers using lethal doses of these antioxidants in the rabbit were apparent. The antioxidants had no effect on the concentration in the liver of the total and esterified cholesterol, total lipid or on the composition of the polyunsaturated fatty acids. However, as the liver weight was increased by BHA or BHT the absolute lipid content of the liver was increased.

INTRODUCTION.

It has been shown (Day, Johnson, O'Halloran and Schwartz, 1959) that the ingestion of the antioxidant butylated hydroxy toluene[4] (BHT) by rats elevates the serum cholesterol and phospholipid levels and in addition (Brown, Johnson

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[4] Tenox BHT 2:6 di-t-butyl-p-cresol.

and O'Halloran, 1959) when BHT was fed in conjunction with a 20 p.c. lard supplement to the stock diet of the rat it did not conform to the generally accepted criteria (World Health Organisation, 1958) for safety as an additive to foodstuffs for human consumption. On the other hand, the antioxidant butylated hydroxy anisole[5] (BHA) in a 10 p.c. lard supplement conformed to these criteria.

Denz and Llaurado (1957) attributed the death of rabbits given large doses of BHA and BHT to an excessive loss of electrolytes, especially sodium, in the urine which results in increased urinary aldosterone excretion with depletion of lipid in the zona glomerulosa of the adrenal.

The present study was undertaken to compare the effects at different dietary levels in the rat of BHA, BHT and a third antioxidant, propyl gallate[6] (PG), in a 20 p.c. lard supplement to the stock diet on the growth, liver and adrenal weights, liver, adrenal and serum cholesterol levels, polyunsaturated fatty acid composition of the total liver lipid, serum sodium and histological appearance of the adrenal.

Experimental.

The stock diet, the method of preparation of the experimental diets and the general procedure for the care of the animals have been described previously (Johnson, O'Halloran and Hewgill, 1958). The stock diet in the present work was supplemented with 20 p.c. lard to which were added increasing amounts of antioxidant so that the diets contained 0, 0·1, 0·2, 0·3, 0·4, 0·5 p.c. (dry weight) antioxidant. The antioxidant was added at the expense of 0·5 p.c. cellulose in the control diet, i.e., the 0 p.c. antioxidant level.

Litters of the Norway Hooded strain of rat were culled so that the litters contained three males and three females. At weaning (28 days) a litter was randomly allocated to one of the dietary concentrations of BHT. Two litters were placed on each experimental diet, the animals being caged in pairs with the sexes segregated. This procedure was repeated with the two remaining antioxidants.

Six weeks later the rats were fasted overnight, anaesthetized with ether and blood was withdrawn by cardiac puncture. The animals were killed by further anaesthetization. The liver was removed, dried with filter paper, and weighed. The left adrenal was removed and weighed after any extraneous tissue had been dissected away. The right adrenal was fixed in 10 p.c. formol saline.

Ethanol-ether (3:1) extracts of the sera were made. The liver lipids were extracted with petroleum ether (BP 40-60° C.) in a Waring Blendor according to the method of Thompson, Ganguly and Kon (1949). The left adrenals of the males and females of one litter were separately pooled and extracted for two hours in a micro-soxhlet apparatus using a 5:1 ethanol-ether solvent.

The total cholesterol content of the lipid extracts was determined by the method of Zlatkis, Zak and Boyle (1953). The free cholesterol was precipitated as the digitonide which was centrifuged off, washed with acetone and analysed by the method of Zlatkis *et al.* (1953). The ester cholesterol was calculated by difference.

The total lipid content of the liver extracts was determined gravimetrically and the polyunsaturated fatty acid composition of the total liver lipids was determined by a modi-

[5] Tenox BHA approximately 85 p.c. 3-t-butyl-4-hydroxy anisole and 15 p.c. 2-t-butyl-4-hydroxy anisole.
[6] Naarden PG.

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fication of the method of Davenport, Birch and Ryan (1956) using the potassium t-butoxide reagent.

Serum sodium was estimated by flame photometry. Some haemolysis occurred during the collection of the blood so that no attempt was made to estimate serum potassium.

Haemtoxylin-eosin and sudan IV staining were carried out on sections of the right adrenals.

RESULTS.

Weight and histological studies.

The effect of six weeks' feeding of the three antioxidants on the weight of the rat, expressed logarithmically, is shown in Fig. 1. The experimental design introduced a genetic effect which led to some variations between litters; despite this, as shown previously (Brown *et al.*, 1959), BHT significantly reduced the growth rate of the rats, especially the males. BHA and PG had no significant

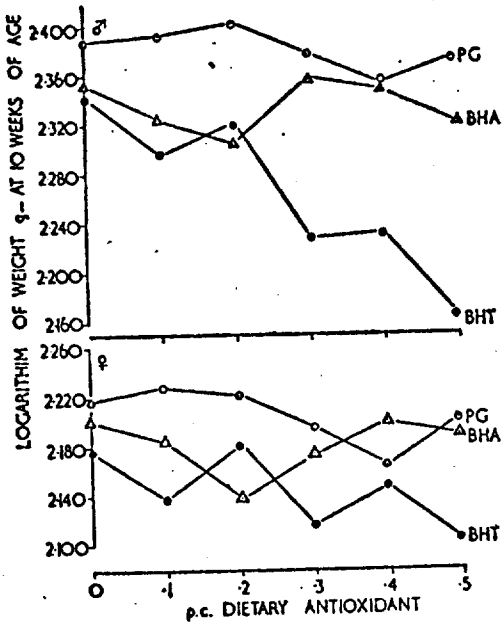

Fig. 1. The effect of six weeks' ingestion of a diet containing various levels of three antioxidants on the log. weight of rats placed on the diets at weaning.
1. Points shown are means of six observations.
2. L.S.D. ($p = 0{\cdot}05$) is the least significant difference for comparison of treatment means (error mean square on 24 degrees of freedom). BHT = 0·054, BHA = 0·036, PG = 0·029.

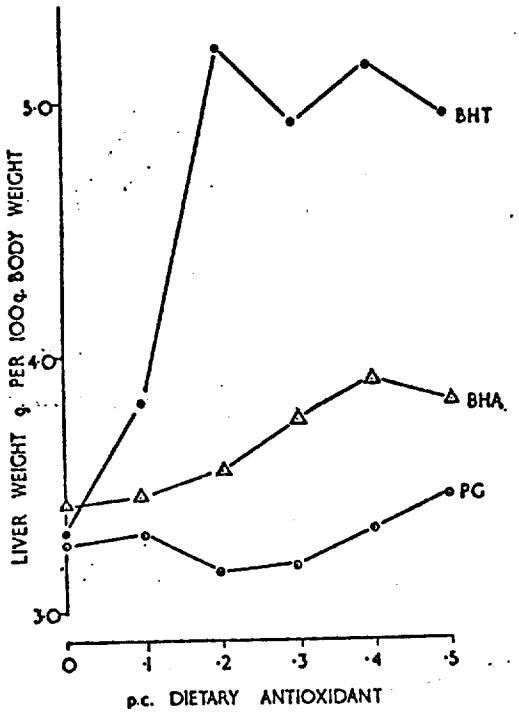

Fig. 2. The effect of three antioxidants on the relative liver weight of the rat.
1. Points shown are means of 12 observations, six male and six female.
2. L.S.D. ($p = 0{\cdot}05$). BHT = 0·444, BHA = 0·225, PG = 0·160.

effect on the growth rate of the animals even when 0·5 p.c. was present in the diet.

BHA and BHT produced increases in the liver weight-body weight ratio, while PG had no effect (Fig. 2). With BHT the relative increases were more marked and the absolute weight of the liver was increased.

BHA and BHT produced increases in the weight of the male left adrenal relatively to the body weight (Fig. 3) but had no consistent effect on the female left adrenal. PG had no effect on the relative weight of either the male or female adrenal.

No histological changes attributable to the treatments could be detected in the adrenal. There was no depletion of lipid staining of the zona glomerulosa or any increase in cytoplasmic basophilia as shown by Denz and Llaurado (1957) in the adrenals of rabbits given large doses of BHA or BHT.

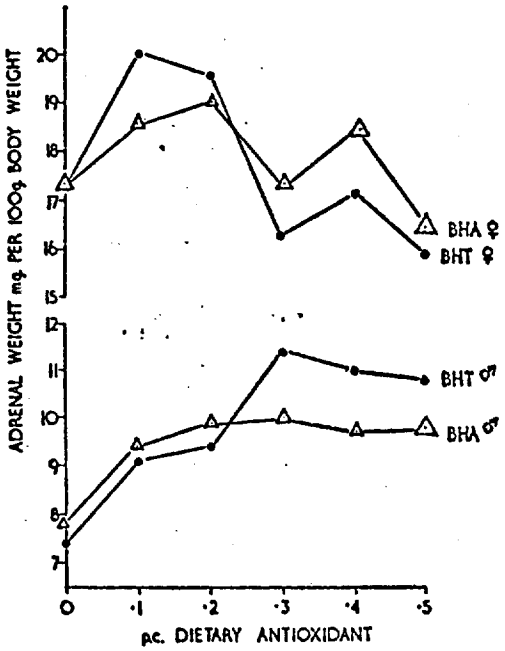

Fig. 3. The effect of BHA and BHT on the relative left adrenal weight of the rat.
1. Points are means of six observations.
2. L.S.D. ($p = 0·05$). BHT = 3·37, BHA = 1·57.

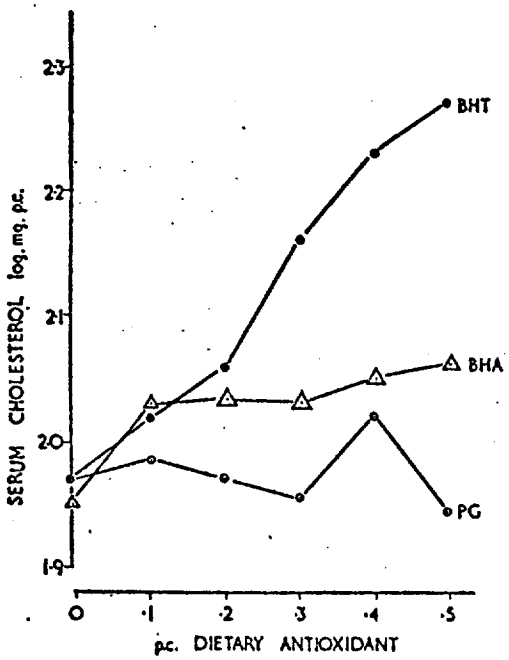

Fig. 4. The effect of three antioxidants on the log. serum cholesterol levels of the rat.
1. Points are means of 12 observations.
2. L.S.D. ($p = 0·05$). BHT = 0·049, BHA = 0·041, PG = 0·039.

Chemical Determinations.

Sera.

The effect of the three antioxidants on the mean serum cholesterol levels, expressed logarithmically, of both male and female rats is shown in Fig. 4. The level of serum cholesterol was directly proportional to the level of dietary BHT.

The mathematical expression of the straight line response between these two variables was $y = 1 \cdot 9557 + 0 \cdot 6214x$. BHA also produced a significant increase in the level of serum cholesterol but this effect was not further increased significantly by increasing the dietary level of BHA above 0·1 p.c. PG did not have any significant effect on the serum cholesterol, with the exception of a diet containing 0·4 p.c. PG. This inconsistent effect was produced by the abnormally high level of serum cholesterol in three male rats of one litter.

The increase in total serum cholesterol produced by BHA and BHT was a result of increases in both the free and esterified fractions. However, as there was a greater increase in the free cholesterol fraction the percent. of esterified cholesterol in the total serum cholesterol fell (Fig. 5). PG had no effect on this value.

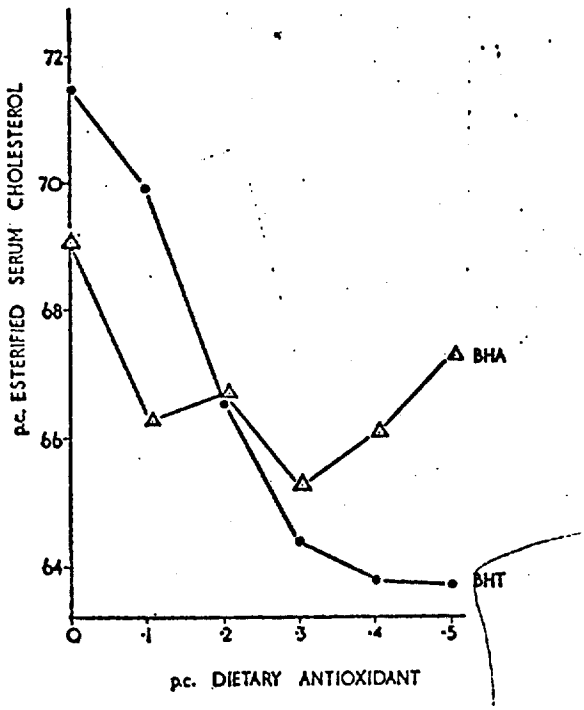

Fig. 5. The effect of BHT and BHA on the percent. esterified serum cholesterol.
1. Points are means of 12 observations.
2. L.S.D. ($p = 0 \cdot 05$). BHT = 2·5, BHA = 1·8.

The serum level of sodium was not affected by the inclusion of the antioxidants in the diet.

Adrenal cholesterol.

BHT produced a significant increase in the concentration of total adrenal cholesterol and there was a strong trend towards an increase in the percent. esterified adrenal cholesterol with increased dietary level of BHT (Figs. 6 and 7). BHA and PG had no significant effect on either of these constituents.

Liver lipids.

None of the antioxidants produced any significant changes in the concentration of the total or percent. esterified liver cholesterol, total liver lipid, or the $K_{1\,cm.}^{0.1\,p.c.}$ (extinction coefficient) value at the five wavelengths at which the polyunsaturated fatty acids are measured. However, as BHA and BHT produced increases in the relative weight of the liver, the absolute amounts of cholesterol and total lipid per rat liver were elevated.

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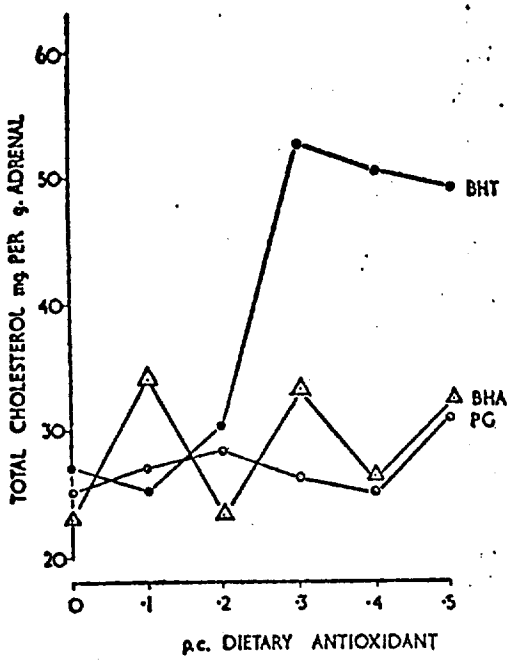

Fig. 6. The effect of three antioxidants on the concentration of total cholesterol of the rat left adrenal.
1. Points are means of four observations, being the pooled adrenals of three rats.
2. L.S.D. ($p = 0{\cdot}05$). BHT = 16·7, BHA = 27·2, PG = 10·9.

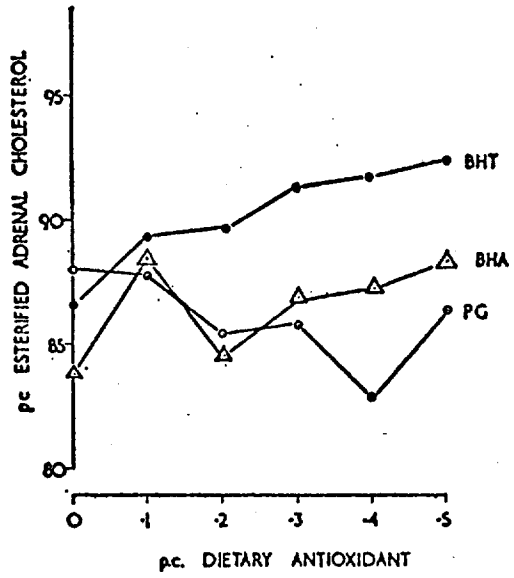

Fig. 7. The effect of three antioxidants on the percent. esterified adrenal cholesterol.
1. Points are means of four observations, being the pooled adrenals of three rats.
2. L.S.D. ($p = 0{\cdot}05$). BHT = 8·2, BHA = 8·3, PG = 5·3.

Urinary metabolites.

During food consumption studies the urine of the animals was absorbed on blotting paper placed in tin containers. The absorbed urine of the animals fed BHT and PG changed colour. That of the BHT animals turned bright pink and that of the PG animals turned purple. When the urine was collected in glass containers no such change occurred and these colours probably arose out of the metal catalysed oxidation of urinary metabolites of the two antioxidants (Dacre, 1961, and Booth *et al.*, 1959).

DISCUSSION.

The effects of BHA and BHT on the growth and liver weight of the rat confirm our previous finding (Brown *et al.*, 1959) that there is a greater hazard involved in using BHT as an additive for use in human foodstuffs than in using BHA. Neither BHA nor PG produced any significant deleterious effects on the growth rate of rats, giving confirmation to the work of Brown *et al.*

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(1959), Wilder and Kraybill (1948), Graham *et al.* (1954), Graham and Grice (1955) and Orten, Kuyper and Smith (1948). BHA and PG thus fulfil one of the requirements for safety for use in human foods.

In accordance with our previous findings (Brown *et al.*, 1959) a sex difference in the effect of BHT was noted. BHT had a greater deleterious effect on the growth of the male than of the female rat and BHT and BHA produced increases in the relative adrenal weight of the male but not the female. Differences in the antioxidant potency of the male and female hormones have previously been observed: Meyer and McShan (1948) and Schuler and Meier (1956) showed that the oestrogens, in contradistinction to the androgens, can act as antioxidants, while Hood *et al.* (1950) showed that the fat from chickens given oestrogen implants was more stable than the fat from control animals.

The rat chronically fed a diet containing relatively less BHA or BHT than the acute doses (1 gm. per day) given to the rabbit by Denz and Llaurado (1957) did not show the marked histological changes of the adrenal produced in that species. The increases in the relative weight of the adrenals in the case of male rats fed BHA and BHT may well be a reflection of the changes produced by the dose level of the antioxidants which led to the death of the rabbits.

The straight line relationship between dietary BHT and the serum cholesterol is indicative of a cause and effect relationship and suggests the possibility that dietary levels of BHT normally ingested by man would cause similar but greatly reduced effects on the serum cholesterol. The straight line relationship has also enabled us to use the maximum concentration of BHT, i.e., 0·5 p.c. of the diet, in our later experiments and relate our findings to more physiological levels of dietary BHT. The lack of any further elevation of serum cholesterol on increasing the dietary level of BHA above 0·1 p.c. of the diet cannot be easily explained. It may be related to the relative antioxidant potency or ease of absorption of BHT and BHA. It is unlikely that the effects of BHA and BHT can be attributed solely to their antioxidant properties, as PG produced no changes in any of the parameters measured.

The elevated levels of serum cholesterol and the increase in the absolute amount of liver and adrenal cholesterol produced by BHA and BHT could be accounted for by an increased rate of synthesis or decreased breakdown of cholesterol or variations in absorption or excretion. Evidence that the first explanation is more likely will be presented in two succeeding papers (Johnson and Reid; Johnson and Holdsworth, to be published).

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Liver Response Tests. VI. Application to a Comparative Study of Food Antioxidants and Hepatotoxic Agents*

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(*Received* 1 *July* 1965)

Abstract—The toxicological significance of hepatomegaly induced by chemical agents has been studied by comparing the response of the liver after exposure of rats to one of 3 hepatotoxins, or one of 3 food antioxidants, followed by a period of recovery. Changes in the following liver parameters were investigated: relative weight; activities of certain enzymes, assessed biochemically and histochemically; histological picture.

The hepatotoxins were administered by stomach tube daily for 1 wk at 2 dose levels (mg/kg) as follows: carbon tetrachloride (160, 1600; single doses only); ethionine (10, 100) and coumarin (20, 200). The antioxidants, butylated hydroxytoluene (BHT; 3,5-di-*tert*-butyl-4-hydroxytoluene), butylated hydroxyanisole (BHA; 3-*tert*-butyl-4-hydroxyanisole) and propyl gallate (PG) were similarly administered at graded dose levels ranging from 50–500 mg/kg.

Liver enlargement was only conspicuous with coumarin, BHT and BHA. Carbon tetrachloride, coumarin and ethionine brought about a decrease in glucose 6-phosphatase with a simultaneous increase in glucose 6-phosphate dehydrogenase activity. BHT affected both enzymes, but only at a dose of 500/mg/kg, whereas BHA and PG did not alter the activity of either enzyme. Substantially the same conclusions were drawn from histochemical studies, which also revealed that the loss of glucose 6-phosphatase activity was predominantly perilobular.

The histological alterations in the liver consisted mainly of fatty change, most striking in the case of carbon tetrachloride, less so with ethionine and PG, very slight with coumarin and BHT, but not seen with BHA. There was an unexpected incidence of abnormal mitotic figures in the group treated with PG which were still present after 14 days' recovery but had completely disappeared by day 28.

After recovery, a clear distinction could be drawn between the ready reversibility of the liver response to the food antioxidants, compared with the greater persistence of the effects on the liver induced by hepatotoxins; for example, after 14 days the depressed activity of glucose 6-phosphatase was still manifest, only returning to the normal level by day 28. Fatty change induced by carbon tetrachloride and coumarin persisted, though at a reduced level, throughout the 28-day recovery period in contrast to the rapid disappearance of this effect elicited by BHT and PG.

Further evidence to show that, as a rule, hepatotoxic agents do not stimulate processing enzyme activity has been provided by a study demonstrating the lack of effect of 7 daily doses of 200 mg coumarin/kg on hexobarbitone oxidase, nitroanisole demethylase and aminopyrine demethylase in the liver of rats.

INTRODUCTION

Previous papers in this series have been concerned with the criteria of liver response to chemical agents. It was shown that depression of the activity of glucose 6-phosphatase, usually but not invariably associated with a simultaneous increase in glucose 6-phosphate dehydrogenase, characterized the action of several hepatotoxic compounds tested (Feuer, Golberg & Le Pelley, 1965 a,b). On the other hand, the significance to be attributed to

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hepatomegaly remains uncertain. A previous paper from this laboratory (Gilbert & Golberg, 1965) has served to introduce the problem presented by liver enlargement, developing in the absence of histopathological or other evidence of liver damage, as a result of the administration of test compounds to animals. In order to make progress in resolving the differences in interpretation that frequently arise in such cases it is necessary to apply more sensitive tests for liver damage, under conditions in which the test material is compared side by side with known hepatotoxic agents. This is what has been attempted here, on the basis of a 1-wk test applying the biochemical and histochemical methods used in earlier work.

In the experiments to be described, the choice of materials investigated was dictated by the desire to include known hepatotoxins with widely different effects and, as far as possible, known mechanisms of action. The 3 compounds selected were carbon tetrachloride, ethionine and coumarin. Three food antioxidants were used for purposes of comparison, 2 of them capable of inducing liver enlargement at high doses, namely butylated hydroxytoluene (BHT; 3,5-di-*tert*-butyl-4-hydroxytoluene) and butylated hydroxyanisole (BHA; 3-*tert*-butyl-4-hydroxyanisole) (Gilbert & Golberg, 1965; Gaunt, Feuer, Fairweather & Gilbert, 1965) while the third, propyl gallate (PG), does not appear to possess this property (Joint FAO/WHO Expert Committee on Food Additives, 1962).

The detailed studies undertaken have included follow-up experiments during a prolonged phase of recovery from the effects of administration of these 3 food antioxidants and 2 of the hepatotoxins, coumarin and carbon tetrachloride. Finally, brief reference is also made to the question of the effect of hepatotoxic compounds on the activities of liver processing enzymes.

EXPERIMENTAL

Materials. Samples of BHA and BHT were obtained from the same sources and conformed to the same specification as described by Gilbert & Golberg (1965). PG was supplied by Hopkin & Williams Ltd., Chadwell Heath, Essex. Carbon tetrachloride and DL-ethionine were obtained from British Drug Houses Ltd., Poole, Dorset and coumarin from W. J. Bush & Co. Ltd., London.

Animals and diet. Male and female SPF Carworth rats were fed on the same diet as detailed by Gilbert & Golberg (1965) and given water *ad lib.*

Dosage. DL-Ethionine, coumarin, BHT, BHA and PG were administered by stomach tube daily for 1 wk at graded dose levels (as stated in Results section) in 5 ml arachis oil/kg body weight. Carbon tetrachloride was injected subcutaneously as a single dose in 5 ml arachis oil/kg. Control groups received equivalent amounts of arachis oil by the appropriate route.

Conduct of experiment. The animals were killed 24 hr after the final dose. Histological examination and biochemical measurements were carried out on the livers of all animals. Histochemical tests were performed on the livers of animals at the lowest and highest dose levels. Histological examination was performed only on the right lobe of the liver.

Frozen liver sections were used to assay the activities of the following enzymes: acid phosphatases (Gomori, 1950; Barka & Anderson, 1962), glucose 6-phosphatase (Wachstein & Meisel, 1956), glucose 6-phosphate dehydrogenase (Pearse, 1960) and esterase (Holt, 1952).

The preparation of liver homogenates and the biochemical measurement of glucose 6-phosphatase and glucose 6-phosphate dehydrogenase was carried out according to

Table 1. *Effect of hepatotoxic agents and BHA, BHT and PG on relative weight and enzyme activities of rat liver*

Compound	Dose (mg/kg/day)	Relative liver weight (%)		Glucose 6-phosphatase		Glucose 6-phosphate dehydrogenase	
		Males	Females	Males	Females	Males	Females
Carbon tetrachloride (single dose)	0	4·80±0·11	4·60±0·09	1·98±0·05	1·56±0·05	0·36±0·04	0·44±0·04
	160	4·94±0·10	4·45±0·07	1·66±0·14*	1·40±0·10	0·35±0·02	0·64±0·07*
	1600	5·05±0·12	4·89±0·15	1·49±0·10**	1·17±0·03***	0·64±0·08**	0·84±0·08**
Ethionine	0	5·12±0·15	4·59±0·17	1·88±0·07	2·02±0·08	0·38±0·03	0·49±0·04
	10	5·09±0·18	5·43±0·18**	1·94±0·06	1·91±0·13	0·53±0·05*	0·56±0·25
	100	4·51±0·21*	5·03±0·12	1·44±0·04***	1·26±0·07***	0·62±0·02***	1·10±0·33***
Coumarin	0	4·04±0·41	4·41±0·18	1·53±0·07	1·85±0·12	0·58±0·07	0·51±0·09
	20	4·53±0·17	4·49±0·29	1·31±0·06*	1·57±0·04	0·60±0·12	0·58±0·12
	200	4·97±0·21**	5·34±0·13**	1·11±0·12*	1·29±0·08**	1·68±0·11***	0·73±0·03*
BHA	0	5·52±0·08	4·98±0·09	1·55±0·13	1·49±0·46	0·44±0·03	0·45±0·15
	50	5·43±0·15	4·59±0·10**	1·13±0·05*	1·48±0·15	0·28±0·03**	0·76±0·10
	100	6·08±0·25*	5·04±0·45	1·08±0·09*	1·04±0·09	0·47±0·15	0·72±0·07
	200	6·05±0·24*	5·58±0·10**	2·31±0·12**	1·47±0·19	0·44±0·12	0·42±0·06
	500	6·00±0·21*	6·25±0·17***	1·56±0·16	1·30±0·11	0·49±0·09	0·52±0·08
BHT	0	4·36±0·28	4·98±0·36	1·66±0·20	1·56±0·06	0·56±0·02	0·50±0·04
	50	4·58±0·10	5·57±0·13	1·44±0·08	1·46±0·12	0·55±0·05	0·48±0·07
	100	5·74±0·14**	5·49±0·15	1·39±0·02	1·32±0·07*	0·57±0·05	0·49±0·11
	200	5·84±0·12**	6·16±0·22*	1·42±0·06	1·24±0·01**	0·42±0·05*	0·59±0·09
	500	6·77±0·10***	6·15±0·21**	1·31±0·14	0·98±0·12**	1·21±0·15***	1·23±0·09***
PG	0	5·55±0·29	5·81±0·15	1·73±0·06	1·74±0·08	0·27±0·04	0·42±0·04
	50	5·32±0·22	5·43±0·17	1·42±0·13	1·78±0·11	0·36±0·02	0·41±0·05
	100	5·27±0·20	5·25±0·06*	1·78±0·18	1·74±0·05	0·41±0·05	0·53±0·08
	200	5·24±0·39	5·35±0·45	1·64±0·25	1·78±0·09	0·31±0·05	0·61±0·21
	500	5·46±0·44	5·55±0·40	1·78±0·24	1·88±0·18	0·41±0·08	0·53±0·06

Groups of 4–12 rats were given a single subcutaneous dose of carbon tetrachloride or, in the case of other compounds, 7 daily oral doses. Liver weight is expressed as a percentage of body weight and enzyme activity in units representing the decomposition of 1 μmole of substrate/hr/mg protein; results are given as means ±SE. Other details are given in the text. Values marked with asterisks differ significantly from those of controls: * $P<0·05$; ** $P<0·01$; *** $P<0·001$.

Table 2. *Histological and histochemical changes in livers of rats treated with various hepatotoxic compounds or BHT*

Compound	Dose (mg/kg/day)	Group size		Fatty change		Histochemical change: Glucose 6-phosphatase		Glucose 6-phosphate dehydrogenase		Non-specific esterase		Acid phosphatase: Gomori		Acid phosphatase: Barka	
		M	F	M	F	M	F	M	F	M	F	M	F	M	F
Carbon tetrachloride (single dose)	0	4	4	N	N	N	N	N	N	N	N	N	N	N	N
	160	6	6	I (5)	I (1)	N	N	N	N	N	N	N	N	D (2)	N
	1600	6	6	I (6)	I (6)	D (6)	D (6)	I (6)	I (4)	D (6)	D (5)	D (6)	D (2)	D (6)	D (2)
Ethionine	0	4	4	N	N	N	N	N	N	N	N	N	N	N	N
	10	5	6	N	I (6)	N	D (1)	N	N	N	N	N	N	N	N
	100	5	6	I (2)	I (4)	D (5)	D (6)	N	I (5)	N	D (3)	N	D (4)	N	N
Coumarin	0	4	4	N	N	N	N	N	N	N	N	N	N	N	N
	20	5	4	I (2)	I (2)	N	N	N	N	N	N	N	D (1)	N	N
	200	5	6	N	I (3)	D (5)	D (6)	I (4)	I (6)	N	N	N	D (5)	D (5)	D (3)
BHT	0	4	4	N	N	N	N	N	N	N	N	N	N	N	N
	50	6	6	I (1)	I (1)	D (2)	N	I (3)	N	N	N	N	N	N	N
	500	6	6	I (2)	N	D (4)	D (2)	I (4)	N	D (3)	N	D (4)	N	D (4)	D (1)

M = Males F = Females N = No change I = Increased D = Decreased.
Figures in parentheses indicate number of animals affected.

Feuer *et al.* (1965a). Modified methods developed in our laboratory (Gilbert & Golberg, 1965) were used for the determination of hexobarbitone oxidase, nitroanisole demethylase and aminopyrine demethylase activities in liver homogenates.

RESULTS

Observations on liver weight and enzyme activities

Administration of a single dose of carbon tetrachloride or of 7 daily doses of the other compounds studied brought about retardation of growth at the highest levels of administration of all compounds, with the exception of BHA which did not appear to influence growth.

Values for relative liver weight are shown in Table 1. It is clear that hepatomegaly was not a regular outcome of the action of hepatotoxins or antioxidants. BHA and BHT caused an increase in liver weight in males at doses of 100, 200 and 500 mg/kg, but in females only at 200 and 500 mg/kg. As expected, PG did not bring about hepatomegaly.

Comparing the results of biochemical measurements, the highest levels of hepatotoxins all gave rise to a decrease of glucose 6-phosphatase activity and an increase in glucose 6-phosphate dehydrogenase. BHA and PG produced no change, while BHT had an effect on the two enzymes at a dose of 500 mg/kg and at lesser doses, particularly in females, glucose 6-phosphatase only was affected.

Histopathological and histochemical findings

These are summarized in Tables 2 and 3.

Fatty change was the principal histological finding, with hydropic degeneration in the case of carbon tetrachloride. Although the presentation of results in tabular form takes note only of the number of animals affected, the degree of change varied markedly in the 4 groups. Predominantly centrilobular fatty change was most pronounced in the case of

Table 3. *Degree of fatty change in livers of rats treated with BHA, BHT or PG*

Compound	Dose* (mg/kg/day)	Group size		Fatty change	
		Males	Females	Males	Females
BHA	0	4	4	N	N
	50	4	4	N	N
	100	4	4	N	N
	200	4	4	N	N
	500	4	4	N	N
BHT	0	12	12	N	N
	50	6	6	I (1)	I (1)
	100	4	6	I (2)	N
	200	4	6	I (2)	N
	500	4	6	I (2)	N
PG	0	4	4	N	N
	50	4	4	N†	N†
	100	4	4	I (1)†	I (3)†
	200	3	4	I (2)†	I (1)†
	500	4	4	I (2)†	I (2)†

N = No change. I = Increased, with number of animals affected in parentheses.
*7 daily oral doses.
†Abnormal mitotic figures in liver (Fig. 1).

Table 4. *Relative weight and enzyme activities of livers of rats recovering for up to 28 days after treatment with carbon tetrachloride, coumarin, BHA, BHT or PG*

Compound	Relative liver weight (%)		Glucose 6-phosphatase		Glucose 6-phosphate dehydrogenase	
	Males	Females	Males	Females	Males	Females
			After 14 days' recovery			
Arachis oil, control	4·84±0·27	4·67±0·38	2·12±0·03	1·26±0·12	0·33±0·07	0·46±0·06
Carbon tetrachloride	4·32±0·20	4·28±0·19	1·85±0·08**	1·55±0·09	0·37±0·13	0·61±0·05
Coumarin	4·15±0·12*	4·15±0·10	1·85±0·08**	1·33±0·08	0·35±0·12	0·47±0·05
BHA	5·27±0·28	4·95±0·34	1·96±0·16	1·22±0·14	0·57±0·06*	0·49±0·09
BHT	5·46±0·17	5·28±0·14	2·23±0·07	0·97±0·06*	0·55±0·11	0·33±0·03
PG	4·87±0·16	5·13±0·14	2·47±0·31	1·12±0·05	0·62±0·05**	0·35±0·06
			After 28 days' recovery			
Arachis oil, control	5·09±0·21	5·17±0·10	1·31±0·14	1·31±0·17	0·17±0·02	0·74±0·05
Carbon tetrachloride	4·59±0·22	4·70±0·13*	1·33±0·17	1·28±0·07	0·22±0·03	0·68±0·19
Coumarin	5·04±0·15	4·76±0·11*	1·43±0·03	1·35±0·05	0·24±0·05	0·73±0·08
BHA	4·74±0·32	4·90±0·18	1·25±0·11	1·30±0·08	0·19±0·02	0·66±0·11
BHT	4·95±0·23	5·14±0·23	0·99±0·17	1·43±0·25	0·23±0·06	0·72±0·05
PG	4·34±0·20*	4·84±0·14	1·05±0·02	1·73±0·10	0·19±0·06	0·50±0·15

Groups of 4–8 rats were given a single subcutaneous dose of carbon tetrachloride (1600 mg/kg) or, in the case of other compounds, 7 daily oral doses (coumarin at 200 mg/kg; BHA, BHT or PG at 500 mg/kg). Liver weight is expressed as a percentage of body weight and enzyme activity in units representing the decomposition of 1 μmole of substrate/hr/mg protein; results are given as means ±SE. Other details are given in the text. Values marked with asterisks differ significantly from those of controls: * $P<0·05$; ** $P<0·01$; *** $P<0·001$.

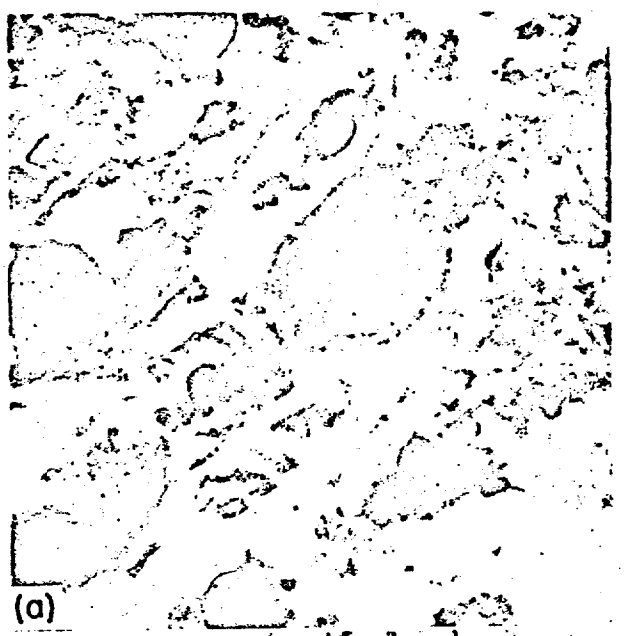

FIG. 1a. Unequal division of chromatin in a liver cell of a rat killed after 14 days' recovery following 7 daily doses by stomach tube of 500 mg PG/kg. Haematoxylin and eosin × 1200.

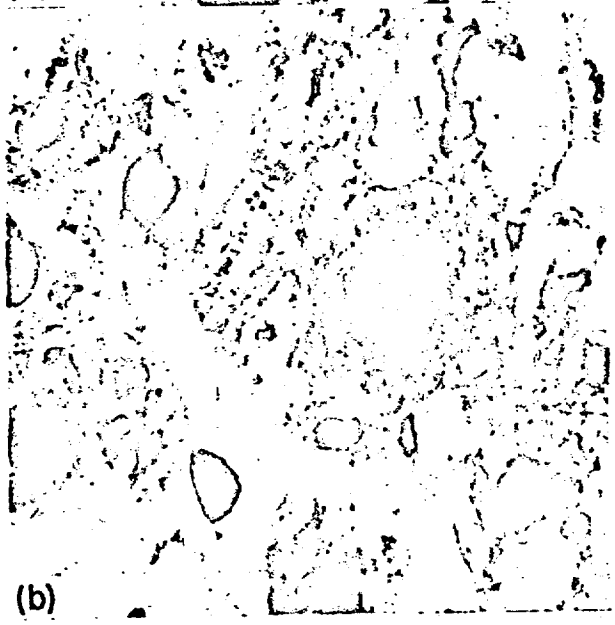

FIG. 1b. Irregular disposition of chromosomes in a liver cell of another rat killed at 14 days' recovery following 7 daily doses by stomach tube of 500 mg PG/kg. Haematoxylin and eosin × 1200.

carbon tetrachloride, less so with ethionine and PG and was only slight with coumarin and BHT. No fatty change was observed at any level of BHA. With BHT and PG there was a slight increase in liver fat in some animals but it was not dose dependent and is not regarded as significant. However, with all doses of PG there was a striking incidence of abnormal mitotic figures which were not present in the control animals nor in any of the other groups (Figs. 1a,b).

Histochemical assessments of enzyme activity were carried out only in the case of the 3 hepatotoxic agents and of BHT. In general, the histochemical findings bear out the conclusions reached on the basis of biochemical measurements. Glucose 6-phosphatase activity was reduced perilobularly at the highest dose levels of all 4 compounds. In the case of glucose 6-phosphate dehydrogenase, male rats on ethionine and females on BHT showed no effect; but otherwise the enzyme activity was increased at the highest levels of dosage. Only in rats given carbon tetrachloride did non-specific esterase show a distinct reduction in activity.

The two methods used to demonstrate acid phosphatase activity gave concordant results, except for the striking difference in males treated with the high dose of coumarin. By both

Table 5. *Degree of fatty change in livers of rats recovering for up to 28 days after treatment with carbon tetrachloride, coumarin, BHA, BHT or PG*

Compound	Dose* (mg/kg/day)	Group size		Fatty change	
		Males	Females	Males	Females
At the end of administration (from Tables 2 and 3)					
Arachis oil, control	—	18	18	N	N
Carbon tetrachloride (single dose)	1600	6	6	I (6)	I (6)
Coumarin	200	4	4	N	I (3)
BHA	500	4	4	N	N
BHT	500	4	6	I (2)	N
PG	500	4	4	I (4)†	I (4)†
After 14 days' recovery					
Arachis oil, control		6	6	N	N
Carbon tetrachloride		6	6	I (6)	I (5)
Coumarin		6	6	I (4)	I (5)
BHA		3	3	I (1)	I (1)
BHT		6	6	I (1)	I (2)
PG‡		6	6	I (3)†	I (6)†
After 28 days' recovery					
Arachis oil, control		6	6	N	N
Carbon tetrachloride		6	6	I (3)	I (4)
Coumarin		6	6	I (3)	I (3)
BHA		3	3	N	N
BHT		6	6	N	N
PG		6	6	N	I (1)

N=No change. I=Increased, with number of animals affected in parentheses.

*7 daily oral doses.

†Abnormal mitotic figures in liver (Fig. 1).

‡At the 500 mg/kg level of PG, extensive fatty change was observed immediately after dosage in contrast to the mild effect seen after 14 days.

methods the decrease of enzyme activity was predominantly centrilobular. It is clear, however, that none of the agents tested produced consistently uniform effects on acid phosphatase in the majority of animals of both sexes.

Effects of recovery

Comparison of Table 1 with Table 4 reveals the effects of 14 and 28 days' recovery on the relative liver weight and enzyme activities measured biochemically. The principal finding at 2 wk was the persistent lowering of glucose 6-phosphatase activity in males that had been treated with carbon tetrachloride or coumarin. There was a tendency for BHA and PG to produce an elevation of glucose 6-phosphate dehydrogenase in males, but this change is probably of no significance. By 28 days no biochemical changes could be seen in contrast to the return to normal by day 14 of relative liver weights.

The only histological changes seen are summarized in Table 5. The increased incidence of fatty change in the groups killed after 14 days' recovery was associated with particularly striking amounts of fat present in the livers of rats previously treated with carbon tetrachloride or coumarin. This change persisted to a considerable degree in animals from the same groups killed after 28 days' recovery, but in the antioxidant-treated groups there was reversion to the normal pattern.

The abnormal mitotic figures seen immediately after treatment persisted through the first 14 days of recovery but were no longer manifest after 28 days.

Effects on processing enzymes

Following preliminary tests of the effect of hepatotoxins on liver microsomal processing enzymes, a formal study was carried out with coumarin (Table 6). This confirmed the earlier impression that, under the conditions employed, hepatotoxic agents do not stimulate the activity of the processing enzymes hexobarbitone oxidase, nitroanisole and aminopyrine demethylase.

Table 6. *Relative weight and activity of processing enzymes in livers of rats treated with coumarin*

Compound	Sex	Relative liver weight (%)	Enzyme activity (μmoles/hr/g liver)		
			HO	NADM	APDM
Arachis oil, control	Male	4·85±0·17	1·96±0·35	2·21±0·56	0·066±0·024
Coumarin		5·40±0·15*	2·58±0·45	2·42±0·18	0·066±0·009
Arachis oil, control	Female	4·43±0·20	1·61±0·38	1·56±0·17	0·121±0·023
Coumarin		5·85±0·10***	1·65±0·38	1·67±0·19	0·131±0·022

Groups of 4 rats were given 7 daily oral doses of coumarin, 200 mg/kg in 5 ml arachis oil/kg; results are given as means ±SE. Values marked with asterisks differ significantly from those of controls: * $P<0·05$; *** $P<0·001$.

DISCUSSION

Hepatic dysfunction and processing enzymes

It has long been known that liver damage prolongs the action and increases the toxicity of many compounds, including barbiturates (Maynert & van Dyke, 1949). In the light of

present-day knowledge many of these effects may be attributed to impaired activity, or inadequate induction, of microsomal processing enzymes in the affected liver. In such circumstances the activities of these enzymes have been assessed either by direct measurement or, indirectly, by the persistence, rate of excretion or duration of pharmacological action of some suitable substrate such as a barbiturate.

McLuen & Fouts (1961) made a particular study of impaired drug metabolism in the damaged livers of rabbits in which obstructive jaundice had been induced by bile-duct ligation. There was a substantial fall in processing activity, clearly manifest on the fourth day; the metabolism of all drugs tested was severely reduced by 10–14 days. In alloxan-treated rats, Fouts (1962) demonstrated within 48 hr a significantly decreased capacity of the liver to metabolize hexobarbitone; by 72 hr it had reached a negligible level. This observation is of particular interest in view of the greatly elevated level of glucose 6-phosphatase in the livers of rats given alloxan (Langdon & Weakley, 1955).

Indirect evidence along similar lines has been provided by Glasson & Benakis (1962), who showed that rats treated with carbon tetrachloride excreted a greater proportion of phenobarbitone in unchanged form, over a greatly extended period. A similar observation was recorded by Plaa, Blaker & McGough (1961) in the case of thioridazine in mice whose livers had been damaged with carbon tetrachloride.

The use of barbiturate sleeping time as a reflection of the hepatic level of processing enzymes has revealed that liver cell injury depresses these 'detoxifying' processes, prolonging the sleeping time. Plaa, Evans & Hine (1958) applied this criterion to the assessment of relative hepatotoxicity and Kutob & Plaa (1962a) embodied the measurement of barbiturate sleeping time in a system for the estimation of hepatotoxic potential. Balazs & Grice (1963) correlated liver necrosis induced by dimethylnitrosamine with prolonged pentobarbitone sleeping time. Weisburger, Schmehl & Pai (1965) did the same in the case of *N*-hydroxy-*N*-2-fluorenylacetamide, where morphological liver damage was maximal at 48 hr and the liver virtually normal again after 1 wk; yet prolonged sleeping time, which was noted at 24 hr, was still in evidence at 1 wk.

It seems clear therefore that, far from activating liver processing enzymes, liver damage induced by toxic agents and other means serves to depress the function of these enzymes. The work with coumarin described in the present communication gives no indication of increased processing enzyme activity. Nevertheless, before it can be firmly concluded that liver enlargement accompanied by stimulation of processing enzymes is not indicative of hepatotoxicity, some further facts must be taken into account.

The ability of stress (in this instance produced by limb ischaemia) to induce liver processing enzymes, and thereby decrease the blood levels of various drugs, has been demonstrated by Bousquet and his colleagues (Rupe, Bousquet & Miya, 1963; Bousquet, Rupe & Miya, 1965, Driever & Bousquet, 1965). The last authors have shown that this response is dependent on an intact pituitary–adrenal axis. We thus have the factor of endocrine intervention, which may exercise its effect in a number of ways; for instance, Gustafsson & Afzelius (1963) have suggested that several of the changes brought about by dimethylnitrosamine or 2-fluorenamine arise from a potentiation of glucocorticoid action on liver cells. Whatever the reason, it has been observed that some hepatotoxic agents may cause an initial rise in microsomal processing enzyme activity. For example, Trams, Inscoe & Resnik (1961) studied the effects of dietary *N*-2-fluorenylacetamide or *p*-dimethylaminoazobenzene on microsomal enzyme activity in rat liver. The hydroxylation of acetanilide and *N*-demethylation of morphine soon became greatly suppressed, despite an initial spurt

of hydroxylation activity induced by *N*-2-fluorenylacetamide during the first 2 wk. It is probably advisable, therefore, to continue such tests for some weeks in order to permit any early effects that may stem from non-specific stress to wear off.

The nature of the liver damage brought about may also be relevant in connexion with the duration of action of a hepatotoxic agent and its consequent effect on processing enzymes. For example, fatty change due to previous exposure to ethanol has been invoked by Kutob & Plaa (1962b) as the explanation for the prolonged action of chloroform in mice, on the grounds that chloroform partitions into the liver lipids. Balazs & Grice (1963) found that allyl alcohol induced massive liver necrosis associated with an increased level of serum glutamic–pyruvic transaminase but no prolongation of pentobarbitone sleeping time.

Effect of processing enzyme stimulators on glucose 6-phosphatase

Treatment of rats with phenobarbitone in doses sufficient to stimulate the activity of liver processing enzymes has been reported by Remmer & Merker (1963) to increase the activity of glucose 6-phosphatase in the smooth membrane fraction of liver endoplasmic reticulum; in the rough membrane fraction it was unchanged. Orrenius & Ernster (1964), however, found a substantial decrease in the activities of glucose 6-phosphatase and nucleoside di- and triphosphatases, persisting throughout and after the period of phenobarbitone administration.

The results of Orrenius & Ernster (1964) are of particular importance in connexion with the observation reported here that doses of BHT above the threshold for the stimulation of processing enzyme activity (Gilbert & Golberg, 1965) are capable of decreasing glucose 6-phosphatase activity. It is clear that the enzyme measurements employed in this work do not constitute adequate criteria of liver response to enable hepatotoxic agents to be distinguished unequivocally from stimulators of processing enzymes.

Acid phosphatase and other lysosomal enzymes

In earlier publications from this laboratory (Feuer, Golberg & Le Pelley, 1964, 1965a) the value of changes in the activities of lysosomal enzymes was assessed in terms of their ability to indicate incipient liver damage. It was concluded that these changes did not take place at a sufficiently early stage, nor with sufficient regularity, to render them reliable criteria. This, too is the conclusion reached in the present paper. It is in keeping with the findings of Slater & Greenbaum (1965) who followed both activity and latency of lysosomal enzymes, only to find that a number of hepatotoxins produced extensive necrosis without evidence of early lysosomal rupture and only small changes in enzyme activity.

Contribution made by histochemical enzyme assessment

The results presented here also make it clear that histochemistry, as a means of assessing the activity of the enzymes studied, has little to contribute to the recognition of early liver damage. The distribution of loss of enzyme activity within the liver lobule is characteristic and it is possible that the combination of centrilobular loss of acid phosphatase with perilobular loss of glucose 6-phosphatase may have some special diagnostic significance. If so, more work is needed to clarify this relationship.

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Tests de réaction hépatique. IV. Application à une etude comparative d'antioxydants alimentaires et d'agents hépatotoxiques

Résumé—On a étudié la signification toxicologique de l'hypertrophie hépatique produite par des agents chimiques, en comparant la réaction du foie après administration à un groupe de rats d'une hépatotoxine choisie parmi un groupe de trois ou à un antioxydant alimentataire choisi de même parmi un groupe de trois. Ce traitement étant suivi par une période de repos. On étudia les variations des caractéristiques suivantes du foie: poids relatif, activités de certaines enzymes déterminées par la biochimie et par l'histochimie, histologie.

Les hépatotoxines étaient administrées chaque jour par tubage gastrique, pendant une semaine à deux doses différentes (mg/kg) de la façon suivante: tétrachlorure de carbone (160, 1600, à raison d'une dose unique); éthionine (10, 100) et coumarine (20, 200). Les antioxydants: hydroxytoluène butylé (BHT; 3,5-di-*tert*-butyl-4-hydroxytoluène), hydroxyanisol butylé (BHA; 3-*tert*-butyl-4-hydroxyanisol) et gallate propylique (PG) étaient administrés de même à des doses progressives variant de 50 à 500 mg/kg.

Le foie s'hypertrophiait notablement seulement avec la coumarine, le BHT et le BHA. Le tétrachlorure de carbone, la coumarine et l'éthionine avaient pour effet de réduire l'activité de la glucose 6-phosphatase et d'accoître simultanément celle de la glucose 6-phosphate déshydrogénase. Le BHT agissait sur les deux enzymes, mais seulement à la dose de 500 mg/kg, tandis que le BHA et le PG ne modifiaient l'activité d'aucune de ces enzymes. Les études histochimiques conduisirent à peu près aux mêmes conclusions. Elles montrèrent également que la diminution de l'activité de la glucose 6-phosphatase avait surtout un caractère périlobulaire.

Les modifications histologiques du foie étaient principalement des modifications graisseuses, surtout frappantes avec le tétrachlorure de carbone, moins frappantes avec l'éthionine, très légères avec la coumarine, le BHT et le PG, et imperceptibles avec le BHA. D'une façon inattendue, on vit apparaître des figures de mitoses anormales dans le groupe traité au PG. Ces images persistaient après 14 jours de repos, et avaient complètement disparu au 28ème jour de repos.

Après repos, on pouvait faire une nette différence entre le caractère facilement réversible de la réaction du foie aux antioxydants de l'alimentation, comparativement à la plus grande persistance des effets produits sur le foie par les hépatotoxines. Par exemple, la diminution de l'activité de la glucose 6-phosphatase était encore sensible au bout de 14 jours, et cette activité ne redevenait normale qu'au 28ème jour. La modification graisseuse produite par le tétrachlorure de carbone et par la coumarine persistait pendant la période de repos de 18 jours, bien qu'à un niveau réduit, tandis que le même effet produit par le BHT et par le PG disparaissait rapidement.

Une étude, qui a montré que 7 doses journalières de 200 mg de coumarine par kg d'animal ne produisent aucun effet sur l'activité de l'hexobarbitone oxydase, de la nitroanisol déméthylase et de l'aminopyrine déméthylase dans le foie des rats, est encore un indice démontrant qu'en principe les agents hépatotoxiques ne stimulent pas l'activité des enzymes détoxifiant.

Untersuchung der Leberreaktion. VI. Anwendung auf eine vergleichende Untersuchung von Lebensmittel-Antioxydationsmitteln und hepatotoxischen Mitteln

Zusammenfassung—Die toxikologische Bedeutung der durch Chemikalien verursachten Hepatomegalie wurde durch Vergleichen der Leberreaktion von Ratten nach Verabreichung eines von 3 Hepatotoxinen oder eines von 3 Lebensmittel-Antioxydationsmitteln mit anschliessender Erholungsperiode untersucht. Es wurden die Veränderungen der folgenden Leberwerte geprüft: das relative Gewicht; die Aktivität mehrerer Enzyme, mit biochemischen und histochemischen Methoden bestimmt; das histologische Bild.

Die Hepatotoxine wurden mit der Magensonde täglich eine Woche lang in zwei Dosierungen (mg/kg) wie folgt verabreicht: Tetrachlorkohlenstoff (160, 1600; nur Einzelgaben); Äthionin (10, 100) und Cumarin (20, 200). Die Antioxydationsmittel butyliertes Hydroxytoluol (BHT; 3,5-Di-*tert*-butyl-4-hydroxytoluol), butyliertes Hydroxyanisol (BHA; 3-*tert*-butyl-4-hydroxyanisol) und Propylgallat (PG) wurden in gleicher Weise in abgestufter Dosierung von 50–500 mg/kg verabreicht.

Die Lebervergrösserung war nur bei Cumarin, BHT und BHA auffällig. Tetrachlorkohlenstoff, Cumarin und Äthionin verursachten eine Abnahme der Glucose-6-phosphatase bei einer gleichzeitigen Erhöhung der Aktivität von Glucose-6-phosphat-Dehydrogenase. BHT beeinflusste beide Enzyme, aber nur bei einer Dosis von 500 mg/kg, während BHA und PG die Aktivität keines dieser Enzyme veränderten. Im wesentlichen die gleichen Schlüsse liessen

sich aus den histochemischen Untersuchungen ziehen, die auch zeigten, dass die Verminderung der Aktivität von Glucose-6-phosphatase vorherrschend perilobär war.

Die histologischen Änderungen in der Leber betrafen hauptsächlich das Fett, am auffallendsten im Falle von Tetrachlorkohlenstoff, weniger ausgesprochen bei Äthionin, sehr gering bei Cumarin, BHT und PG, und nicht festzustellen bei BHA. Unerwartet war das Auftreten von anomalen mitotischen Gebilden in der mit PG behandelten Gruppe, die noch nach 14-tägiger Erholung vorhanden, aber am 28. Tage verschwunden waren.

Nach der Erholungspause konnte klar zwischen der leichten Reversibilität der Leberreaktion auf Lebensmittel-Antioxydationsmittel und dem längeren Persistieren der durch Hepatotoxine verursachten unterschieden werden. So war zum Beispiel nach 14 Tagen die herabgesetzte Aktivität der Glucose-6-phosphatase noch manifest und kehrte erst bis zum 28. Tage auf den Normalwert zurück Die durch Tetrachlorkohlenstoff und Cumarin verursachten Fettveränderungen persistierten, wenn auch vermindert, während der 28 tägigen Erholungsperiode, im Gegensatz zum raschen Verschwinden dieser Veränderungen, wenn sie durch BHT und PG verursacht worden waren.

Einen weiteren Beweis dafür, dass hepatotoxische Mittel in der Regel nicht die Aktivität der arzneimittelmetabolisierenden Enzyme steigern, lieferte eine Untersuchung, welche die Wirkungslosigkeit 7 täglicher Dosen von 200 mg Cumarin/kg auf Hexobarbitaloxydase, Nitroanisoldemethylase und Aminopyrindemethylase auf die Leber von Ratten zeigten.

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TOXICOLOGY AND APPLIED PHARMACOLOGY 6, 512-519 (1964)

Chronic Feeding Studies of Butylated Hydroxyanisole in Dogs

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Butylated hydroxyanisole (BHA) is defined as "a mixture of 85% or more of the 3-*tert*-butylhydroxyanisole and 15% or less of the 2-*tert*-butylhydroxyanisole" (WHO, 1962). Astill *et al.* (1962) estimated that the human intake of phenolic antioxidants as they are used in fat stabilization is probably less than 0.1 mg/kg daily. The results of several studies of the effects of BHA given at various dosages and dietary levels to rats, rabbits, and dogs are summarized in Table 1. In the last line of the table are given the results of feeding studies in dogs begun in 1954 at the suggestion of Dr A. J. Lehman of the U. S. Food and Drug Administration to supplement the data available at that time on rats and dogs. Although the results were submitted to the U. S. Food and Drug Administration and were made available to the Joint FAO-WHO Expert Committee on Food Additives (WHO, 1962), they have not previously been published. On the basis of all evidence available, the Joint FAO-WHO Expert Committee on Food Additives concluded that in the rat, "The level causing no toxicological effect on feeding over a period approximating to the life span is 0.5% of the diet. An inconstant effect on relative liver weight and bodily gain in one series is disregarded" (WHO, 1962). In the study reported below, groups of three dogs each were given daily doses of BHA up to 100 mg/kg and observed for a period of one year. Even the largest daily dose, which corresponds roughly to 0.3% of the diet, failed to produce detectable injury.

METHODS

The dogs were young adult beagles or beagle-type hounds, most of which weighed about 10 kg. The required amounts of BHA were mixed by hand into the basal ration of Purina Dog Chow. The dry meal was first mixed with water and allowed to stand until the proper consistency had been achieved; the BHA doses were then added. The dogs drank tap water supplied ad libitum. They were in good condition throughout the year, maintained good coats, and except for rare, minor, transient illnesses were in good health during the entire period.

BHA was added to the diet in two forms. Four groups of dogs received BHA in a 50% solution in propylene glycol. The fifth group was given BHA in a solution containing approximately 20% BHA, 6% propylgallate, 4% citric acid, and 70% propylene glycol (Tenox II). To ensure correct diet concentrations, the BHA content of these solutions and of the diets was determined periodically using the colorimetric

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method described below on 1:1000 dilutions in 80% ethanol. All BHA doses were expressed on the basis of the BHA content. In the first five months of the experiments the sample of BHA used contained 70% of 3-*tert*-butyl-4-hydroxyanisole, 29% of 2-*tert*-butyl-4-hydroxyanisole, and 1% of 2-*tert*-butyl-4-methoxyanisole. During the last seven months of the study the BHA contained 96% of 3-*tert*-butyl-4-hydroxyanisole, and 4% of 2-*tert*-butyl-4-hydroxyanisole. These differences were not regarded as important toxicologically. The diets contained sufficient BHA to give the following doses: 0 (control dogs), 0.3, 3.0, 30, and 100 mg/kg/day of BHA as the 50% solution in propylene glycol. In addition, one group of three dogs was given 30 mg/kg/day of BHA in the propylgallate-citric acid-propylene glycol solution.

At autopsy samples of fat, brain, liver, and kidney were taken from several dogs. These were analyzed for BHA content using a modification of the Emmerie-Engel reaction as applied to fat-soluble antioxidants (Astill *et al.*, 1959). Weighed 10-g aliquots from fresh tissue samples were macerated with anhydrous Na_2SO_4 and left overnight under $CHCl_3$. The decanted $CHCl_3$ was evaporated, and the residue was dissolved in ligroin. This was washed thrice with aqueous 80% ethanol and the combined wash solutions were mixed in weak artificial light with ethanolic 0.1% $FeCl_3 \cdot 6H_2O$ and ethanolic 0.1% dipyridyl solutions. After 40 minutes in the dark, absorptions at 515 mμ were read on an Evelyn colorimeter against a reagent blank, with a calibration curve from 5 to 50 μg (reliable limits) of BHA in 80% ethanol. Recoveries of known amounts of BHA from tissues were 70 ± 12%. The lower limit of sensitivity was about 1 μg of BHA.

Urine samples from both control and experimental dogs were assayed for substances giving a positive Emmerie-Engel reaction after extraction. Ten-milliliter portions of urine were shaken with ligroin, and the BHA content of the ligroin determined as in the above ligroin tissue extract. Recoveries of known amounts of BHA from urines were quantitative.

RESULTS AND DISCUSSION

The dogs maintained body weight or gained a little during the year; specifically 11 dogs showed gains of 1 or 2 kg and 6 dogs weighed nearly exactly the same initially and finally (Table 2). One dog, a female given 0.3 mg/kg/day, lost 1.4 kg during the last week of the year, presumably from intercurrent illness.

Urine samples were collected pre-experimentally and again at the end of the year and analyzed for protein and sugar contents. With rare exceptions only normal trace values were found.

Before starting the experiment and at 8 regular intervals during the year, blood samples were taken for hematologic examinations. The hematologic data fell almost without exception in the normal ranges (Table 2). There were no trends, either increases or decreases, with increasing BHA dose.

At the end of the year, the dogs were killed by injecting intravenously a large dose of a soluble barbiturate. During the gross autopsy examination, liver, kidneys, lungs, brain, heart, and spleen were dissected out and weighed. Organ weights neither increased nor decreased consistently with increasing doses of BHA (Table 2). One or two unusually heavy organs were found, for example, a liver, a spleen, or a lung. No obvious reason for their size was discovered. With these exceptions, however, the

TABLE 1
SUMMARY OF CHRONIC TOXICITY STUDIES OF BHA

Species	Number in group	% in diet with normal growth	% in diet with growth retardation	Duration	Pathology	Comment	Reference
Rat	7	—	0.5, 1, 2, 3, 6,[a] 12[a]	6 mo.	Negative	Voluntary food refusal; complete at 6 + 12%. Traces of BHA in body fat only at highest doses	Wilder and Kraybill (1948)
Rat	15–20	0.003, 0.03, 0.06	—	22 mo.	Negative	Every female produced at least 1 litter of young, also raised on same diets 12 weeks without effects noted	Wilder and Kraybill (1948)
Rat	17	0.003, 0.03, 0.06, 0.12	—	21 mo.	"Normal"	Survival lower at 0.12%	Wilder and Kraybill (1948)
Rat	26M, 26F	0.00135, 0.00675[b]	—	1 yr.	Same in low and high	Mortality lower in low diet % group but probably not significant. Normal organ weights	Graham *et al.* (1954)
Rat	13M, 13F	0.01, 0.1[c]	—	2 yr.	Negative	Survival, organ weights, reproductive performance equal to control group	Brown *et al.* (1959)
Rat	20M, 20F	0.001,[d] 0.1, 0.5	0.5[e]	8 mo.	Negative	Survival, reproductive performance equal to control group. At 0.5% liver weights increased	Brown *et al.* (1959)
Dog	4[f]	0.0176,[g] 0.176	0.44,[h] 0.88[i]	15 mo.	Negative at 0.44 or less; liver injury at 0.88	Food consumption less, glucuronide in urine increased, organic sulfates in urine increased. Hematology no effect, urinary protein normal	Wilder *et al.* (1960)

TABLE 1 (*Continued*)

			% in diet with growth				
		% in diet with				Comment	Reference

Rat	13M, 13F	0.01, 0.1[c]	—	2 yr.	Negative	Survival, organ weights, reproductive performance equal to control group	Brown *et al.* (1959)
Rat	20M, 20F	0.001,[d] 0.1, 0.5	0.5[e]	8 mo.	Negative	Survival, reproductive performance equal to control group. At 0.5% liver weights increased	Brown *et al.* (1959)
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TABLE 1 (*Continued*)

Species	Number in group	% in diet with normal growth	% in diet with growth retardation	Duration	Pathology	Comment	Reference
Rabbit	—	—	—[j]	11 days or less	—	Death. Sodium, potassium and aldosterone excretion elevated in urine. Histologic changes in adrenal medulla	Denz and Llavrado (1957)
Dog	3	0.001, 0.01, 0.1, 0.3[k]	—	1 yr.	Negative	Organ weights normal. Hematology normal. Urine sugar and protein normal	Current study

[a] Number of rats per group not given.
[b] Mixed in flour and baked in bread for diets.
[c] BHA in hydrogenated coconut oil stimulated growth.
[d] BHA in unheated lard stimulated growth.
[e] BHA in unheated lard, females, and heated lard, males.
[f] 1M and 3F except high level group 2M and 2F.
[g] Calculations on assumed food consumption give approximately equivalent figures.
[h] One dog.
[i] Three dogs.
[j] Repeated daily 0.5 or 1.0 g doses p.o.
[k] Calculated on the basis of 300 g diet per day.

organ weights, both fresh weights and weights calculated on the basis of body weights, fell in the normal ranges.

There was no storage of BHA in the fat, brain, liver, and kidney samples (Table 2). Most tissues contained a small amount of some substance, a reducing agent, which gave an Emmerie-Engel color reaction. Thus, in the control dogs, values of 0–20 μg of reducing substance expressed as BHA were found per gram of tissue. Substantially the same ranges were found for the dogs given 0.3 mg and those given 30 mg per kilogram per day for a year. Values mostly in the same range but including a few values over 20 μg (specifically, 25–35 μg BHA per gram of tissue) were found for the dogs given 100 mg/kg daily. No significance should be attached to these minute differences. For example, one of the dogs given 100 mg/kg/day with a body weight of about 10 kg received 1 g of BHA per day, or approximately 300 g during the year. If the liver (weighing about 500 g) of this dog contained 30 μg of BHA per gram of fresh tissue, the total BHA in the liver would have been 15 mg or 0.005% of 300 g. There were present enough reducing substances in the entire liver to account for approximately one part in 30,000 of the BHA ingested.

Attempts were made to detect extractable BHA in urines by the Emmerie-Engel reaction. Normal dog urines contained a highly variable amount of reducing substance, viz., 5–70 μg/ml expressed as BHA equivalent. There was no increase in the content of ligroin-extractable reducing substance in the urine of the dogs given the highest dietary level of BHA. This accords with the low values for excretion of unchanged BHA in metabolism studies in the dog and other species (Table 3). Variations in amounts of substances normally present would be sufficient to mask any BHA increases of the order expected when simple extraction procedures were used.

Recent studies of the metabolic fate of BHA in the dog (Astill *et al.*, 1962) using refined techniques are consonant with the failure to detect free BHA in the urine (Table 3).

A comparison of the fate of BHA in various species is given in Table 3. Dogs given a single dose of 350 mg/kg excreted less than half the dose in the urine, about 25% of the dose being thus excreted as *O*-sulfates, with *O*-glucuronide formation accounting for about 5%. A large proportion of the dose (more than half) was found unchanged in the feces. Rats and rabbits excreted the greater part of a single dose, over a wide range of doses, in the urine as the 4-*O*-glucuronide conjugate of BHA. Support for these results in rats was provided by Golder *et al.* (1962), who found that 90% of 0.5 mg/kg intraperitoneal doses of BHA-H^3 could be accounted for within 4 days of dosing as radioactivity in the urine. Man excreted small doses (0.4–0.7 mg/kg) in the urine almost entirely as the *O*-glucuronide and only traces as *O*-sulfates. In this regard the metabolic pattern in man appears to be similar to that in the rat and perhaps in the rabbit, and dissimilar to that in the dog.

At the time of the autopsy examination, samples of a number of tissues were removed and prepared for histologic examination using the customary hematoxylin and eosin stains. Tissues examined include the following: heart, lungs, spleen, stomach, small and large intestine, pancreas, liver, adrenal, kidney, urinary bladder, thyroid, bone marrow, and brain. No pathologic changes were found that were attributed to the administration of BHA. In particular, the livers were normal and none of the abnormalities described by Wilder *et al.* (1960) in puppies given daily doses high

TABLE 2

SELECTED OBSERVATIONS ON DOGS FED VARIOUS AMOUNTS OF BHA FOR ONE YEAR

BHA in diet (mg/kg)	Number and sex	Average body weight (kg)		Average hemoglobin, final (g/100 ml)	Organ wt.:body wt. (g/kg)		Range of BHA tissue contents[a] (μg/g fresh weight)			
		Initial	Final		Liver	Kidney	Fat	Brain	Liver	Kidney
Control	2M, 1F	12.1	14.0	17.6	32	5.4	5–18	15–20	5–20	0–15
0.3	1M, 2F	8.1	8.5	16.7	28	6.8	—	10–23	15–20	10–13
3.0	1M, 2F	11.3	12.1	16.9	30	5.8	—	—	—	—

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Control	2M, 1F	12.1	14.0	17.6	32	5.4	5–18	15–20	5–20	0–15
0.3	1M, 2F	8.1	8.5	16.7	28	6.8	—	10–23	15–20	10–13
3.0	1M, 2F	11.3	12.1	16.9	30	5.8	—	—	—	—
30.0	1M, 2F	10.6	11.4	16.3	38	8.1	0–15	10–25	10–20	10–25
30.0[b]	2M, 1F	9.2	10.0	17.0	37	6.1	—	—	—	—
100	1M, 2F	10.3	11.4	17.3	28	6.3	0–15	10–20	25–35	20–30

[a] Phenolic-type reducing agent.
[b] As Tenox II.

TABLE 3
PRINCIPAL METABOLITES OF BHA (PERCENTAGES OF ORAL DOSES)[a]

Species	Dose (mg/kg)	Glucuronide	Ethereal sulfates	Unconjugated	Demethylation	Hydroxylation	Reference
Dog	350	5.5	23	3.6	+	+[c]	Astill *et al.* (1962)
	100[b]	—	—	—	NI	NI	Current study
Rabbit	250 S	60	12	4	NI	NI	Dacre *et al.* (1956)
	500 R	48	14	1	NI	NI	Dacre *et al.* (1956)
Rat	400	72	14	5	†	—	Astill *et al.* (1960)
	2	72	—	10	NI	NI	Astill *et al.* (1960)
Man	0.4–0.7	27–77	+	1	—	—	Astill *et al.* (1962)

[a] Symbols and abbreviations: NI, no information; S, single; R, repeated; +, demonstrated; —, undetectable; †, only as a minor constituent.
[b] Highest daily dose given for 1 year (see text).
[c] Probable.

enough to induce partial voluntary food restriction (250 mg/kg) were present. A number of the common incidental, unrelated pathologic changes were described such as are found from time to time in beagle dogs of our colony. These are summarized in Table 4.

TABLE 4
SUMMARY OF PATHOLOGIC FINDINGS[a]

Dog no.	BHA in diet	Sex	Lung	GI tract	Gonads	Urinary tract	Other organs
2462	Control	M	H	Fl	A		
2465		F		Fi,G		C, P	
2004		M					LVH
2751	0.3 mg/kg	F		Fl		P	
2752		F		EML		PN	
2664		M			TD	P	
1637	3.0 mg/kg	F					
2031		M	BP				
1929		F	Gr				
2245	30 mg/kg	M		T			
1868		F	Gr			P	
2556		F				P, Gr	
1933	30 mg/kg (in Tenox II)	F				PN	
1985		M				Pr	
1966		M				PN	
2457	100 mg/kg	M			TA		H
2400		F				P	
1717		F		Gr			

[a] Abbreviations of pathologic findings:

A,	adenocarcinoma	*H,*	hemorrhage
BP,	bronchopneumonia	*LVH,*	left ventricular hypertrophy
C,	cystitis	*P,*	pyelitis
EML,	enlarged mesenteric lymph nodes	*PN,*	pyelonephritis
Fi,	fibrous nodule	*Pr,*	prostatitis
Fl,	flatworms	*T,*	tapeworm
G,	gastric mucosa	*TA,*	testicular atrophy
Gr,	granulomata	*TD,*	testicular tubule degeneration

There are no detectable effects on dogs given daily doses of 100 mg/kg of BHA (approximately 0.3% of the diet), which is the upper limit of palatability of diets containing BHA. This confirms for this species the existence of noneffective level roughly comparable to the dietary percentage (0.5%) which failed to give positive toxic effects in rats. For the rat, 0.5% in the diet is equivalent to 250 mg/kg body weight per day. Unconditional acceptance of up to 0.5 mg/kg body weight in the human diet was proposed (WHO, 1962), and conditional acceptance for amounts up to 2.0 mg/kg. The dog data reported herewith support the safety of these estimates.

Groups of three dogs eac
amounts of BHA as a 50%
100 mg/kg. An additional gr
containing about 6% propyl
maintained in every dog exc
no deaths. Urine analyses i
Blood samples taken on nin
weights lay in the normal r
and there was no increase
revealed no evidence of any

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colony. These are summarized in

NDINGS[a]

	Gonads	Urinary tract	Other organs
	A		
		C, P	
			LVH
		P	
		PN	
	TD	P	
		P	
		P, Gr	
		PN	
		Pr	
		PN	
	TA		H
		P	

hemorrhage
. left ventricular hypertrophy
pyelitis
pyelonephritis
prostatitis
tapeworm
testicular atrophy
testicular tubule degeneration

aily doses of 100 mg/kg of BHA
pper limit of palatability of diets
the existence of noneffective level
5%) which failed to give positive
is equivalent to 250 mg/kg body
to 0.5 mg/kg body weight in the
itional acceptance for amounts up
port the safety of these estimates.

SUMMARY

Groups of three dogs each were maintained for one year on diets containing the following amounts of BHA as a 50% solution in propylene glycol: 0 (control group), 0.3, 3.0, 30, and 100 mg/kg. An additional group received a 30 mg BHA/kg as a 20% solution in propylene glycol containing about 6% propylgallate and 4% citric acid. Body weights increased slightly or were maintained in every dog except one. The dogs were in good condition during the year; there were no deaths. Urine analyses initially and at the end showed normal values for sugar and protein. Blood samples taken on nine occasions during the study gave normal hematologic values. Organ weights lay in the normal ranges. There was no storage of BHA in fat, brain, liver, and kidney; and there was no increase in urinary reducing substances. Thorough histologic examinations revealed no evidence of any tissue change that was attributed to the administration of BHA.

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Effect of Feeding Butylated Hydroxyanisole to Dogs

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Groups of weanling Cocker Spaniel pups were fed BHA at levels of 0.0, 5.0, 50.0, and 250.0 mg. per kg. of body weight per day for a 15-month feeding period to see what effect, if any, these subacute levels of BHA might have on the dog. General health and weight gains were good throughout the period. Hemoglobin and blood cell counts were not appreciably affected by feeding BHA at the levels used here. Urine from dogs fed BHA contained higher levels of glucuronates and a higher ratio of total to inorganic sulfates, indicating that BHA excretion was by this route. Microscopic examination of tissue sections at the time of autopsy showed no changes beyond normal variation, except in three animals which had received the highest dosage, in which liver injury had occurred. The results indicate that dogs can ingest BHA for a long period without harm, at a level at least 220 times the maximum allowable level for this antioxidant in lard.

The use of antioxidants to prevent the development of oxidative rancidity in animal fats used in food and feed has become very widespread. Permission for the use of butylated hydroxyanisole (a mixture of 2- and 3-*tert*-butyl-4-methoxyphenols, BHA) alone and in combination with other antioxidants and synergists in animal fats and shortenings containing animal fats at specified levels was granted in 1948 (*2*). Since that time, a large proportion of the edible animal fats produced have been treated with BHA as a preservative. The use of such materials in foods makes it desirable to find out more about the effects of the antioxidant when ingested at high levels over a long period of time. The present study was initiated to study the effects of feeding high levels of BHA to dogs.

Previous and concurrent work using rats to study the acute and chronic toxicity of BHA (*1*) indicated that levels of BHA up to 2% of the diet had no demonstrable effect over a 6-month period, and that the LD_{50} for rats was of the order of 4.1 to 5.0 grams per kg. of body weight. This information served as a guide in selecting levels of BHA to feed to dogs in a study of the chronic toxicity of the antioxidant in this species.

Procedure

Sixteen Cocker Spaniel pups were weaned at 6 weeks of age and within 1 to 2 months after weaning, litter mates were assigned to groups with four pups in each group as follows:

1. Control group, basal ration only.
2. Basal ration plus a low level of BHA which probably would produce no adverse effect. This group received 5 mg. of BHA per kg. of body weight per day.
3. Basal ration plus an intermediate level of BHA which might or might not produce injury. This group received 50 mg. of BHA per kg. of body weight per day.
4. Basal ration plus a high level of BHA which approached the highest level tolerated in the diet. This group received 250 mg. of BHA per kg. of body weight per day.

Each group consisted of one male and three females, except group 4 which had two males and two females.

Dosages were adjusted to changes in feed consumption and body weight to maintain the above levels. The basal ration was composed of toasted corn flakes, ground, 24.60%; toasted wheat flakes, ground, 24.56%; soybean oil meal (50% protein), 17.58%; meat and bone meal (50% protein), 13.80%; lard, 8.00%; fish meal (menhaden), 2.76%; wheat germ meal, 4.60%; dried skim milk powder, 2.30%; vitamins A and D oil (2250 U.S.P. units A and 300 I.C. units D per gram), 0.46%; iodized salt, 0.24%; dried brewer's yeast, 0.46%; and riboflavin supplement (BY-500), 0.74%. The BHA levels necessary to obtain the desired dosages were computed and the necessary amounts dissolved in the lard portion before mixing the diets. The dogs were housed in metal cages with expanded metal floors in a heated room and given their food allotment and fresh water twice daily. Body weight and food consumption records were kept for a 14- to 15-month period. Blood and urine analyses were made three times for each dog — twice during the test and again at the time the dogs were sacrificed for autopsy and histological examination of their tissues. The first two blood samples were drawn from a leg vein, and the third sample was collected by heart puncture at the time the dogs were sacrificed.

Results

Weight Gain and Feed Consumption. Weaning weight, final weight at 16 to 18 months of age, and average daily feed consumption are shown in Table I. No appreciable differences in weight gains in the different groups were noted except in the group 4 receiving the highest level of BHA (250 mg. BHA per kg. of body weight daily). The dogs in this group gained less weight and consumed less feed per day. The lowered feed consumption was due to the fact that BHA has a hot, peppery taste which the dogs do not like and it was difficult to disguise the taste of that amount of BHA in the feed. At lower levels (groups 2 and 3), the dogs consumed the feed without difficulty after building up gradually to those levels over an acclimatization period of a few days. During the course of the feeding period, the level of BHA in the diet had no influence on the age at which maximum weight was reached. Some dogs reached their peak weight at 7 months of age, while others continued to gain until they were sacrificed. In all dogs, minor fluctuations in body weight were noted, particularly after about 7 months of age. At the time of autopsy, dog number 11 in group 3 contained a large amount of adipose tissue in the body, and dog number 15 in group 4 was very thin. However, all dogs in the different groups appeared to be normal in general appearance.

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Table I. Effect of BHA Ingestion by Dogs

Group No.	Dog No.	Litter Source No.	Sex	Weaning Wt., G.	Final Wt., G.	Age at Sacrifice, Mo.	Av. Daily Feed Consumption, G.	Hb, %	Red Blood Cells, Blood 1000	Hemoglobin and Blood Cell Counts at Time of Sacrifice: White P.M.N., cells, no.	Differential White Cells[a]: P.M.N. no.	Lympho. no.	Mono. no.	P.M.E. no.	P.M.B. no.
1	1	IV	M	1500	10,000	16	225	17.5	5640	7,200	68	27	4	0	1
	2	II	F	1250	7,900	18	280	17.7	4960	4,500	81	19	0	0	0
	3	III	F	1150	10,800	18	280	17.3	6180	4,250	65	35	0	0	0
	4	I	F	1100	6,900	18	225	15.0	4870	7,000	75	23	2	0	0
							Av. 252	16.9	5412	5,737	72	26			
2	5	I	M	1450	9,000	18	240	17.0	5520	5,050	79	21	0	0	0
	6	II	F	1350	9,400	18	225	16.0	6200	8,900	69	30	1	0	0
	7	III	F	1200	10,300	18	270	16.7	4930	7,800	74	25	1	0	0
	8	IV	F	1200	6,450	16	255	16.5	6040	8,100	64	36	0	0	0
							Av. 245	16.6	5672	7,462	71.5	28			
3	9	II	M	1750	9,350	18	285	15.0	6760	3,250	83	23	4	0	0
	10	I	F	1450	6,150	18	240	15.2	5920	7,300	82	17	1	0	0
	11	III	F	1300	11,150	18	255	14.5	5530	10,900	72	26	2	0	0
	12	IV	F	1400	5,200	16	240	14.5	7070	13,200	79	18	3	0	0
							Av. 255	14.8	6213	8,681	79	21			
4	13	III	M	900	6,600	18	195	15.0	6670	5,450	78	18	3	1	0
	14	II	F	1400	6,500	18	210	15.0	6456	11,800	75	22	3	0	0
	15	I	F	1175	4,325	18	165	15.5	6310	10,900	79	20	1	0	0
	16	IV	M	1450	6,050	16	240	16.0	7510	7,450	62	33	3	2	0
							Av. 202	15.4	6736	8,900	73.5	25.7			

[a] Differential white cell counts. P.M.N. = polymorphonuclear neutrophils. Lympho. = lymphocytes. Mono. = monocytes. P.M.E. = polymorphonuclear eosinophils. P.M.B. = polymorphonuclear basophils.

Table II. Urinalyses of Dogs Fed BHA

(Mg./100 Ml.)

Group No.	Dog No.	Color	pH Reaction to Nitrazine Paper	Albumin	Sugar	Color Test for BHA in Urine	Glucuronic Acid in Urine[a]	Sulfates/100 Ml. Urine: Total sulfates	Inorganic sulfates	Ethereal sulfates	Ratio of Total to Inorganic Sulfates
1	1	Pale yellow	Alkaline, high	−	±		151.3	10.55	8.95	1.60	1.18:1
	2	Pale yellow	Alkaline	±	−		62.5	9.77	8.16	1.61	1.20:1
	3	Pale yellow	Alkaline, high	−	−		215.0	6.06	4.36	2.30	1.39:1
	4	Pale yellow	Alkaline, high	−	±						
						Av.	142.9	8.79	7.15	1.84	1.26:1
2	5	Yellow	Alkaline, high	±	−		240.0	9.41	7.31	2.10	1.29:1
	6	Pale yellow	Acid, slight	−	+		470.0	10.87	9.23	1.64	1.18:1
	7	Pale yellow	Alkaline	−	−	+	370.0	7.42	6.14	1.28	1.21:1
	8	Yellow	Alkaline, high	−	+	+					
						Av.	360.0	9.23	7.56	1.67	1.33:1
3	9	Pale yellow	Alkaline, high	−	−		117.5	4.45	3.19	1.26	1.40:1
	10	Pale yellow	Alkaline, high	−	±	+	437.5	7.04	4.11	2.93	1.71:1
	11	Pale yellow	Alkaline, high	−	−	+	187.5	6.75	4.34	2.41	1.56:1
	12	Pale yellow	Alkaline, high	−	±	+					
						Av.	247.5	6.08	3.88	2.20	1.56:1
4	13	Yellow amber	Alkaline, high	±	+	+	460.0	6.10	0.59	5.51	10.41:1
	14	Yellow amber	Alkaline, high	−	+		430.0	4.13	0.46	3.67	9.06:1
	15	Pale yellow	Neutral	−	+	+	427.5	5.91	2.22	3.69	2.66:1
	16	Yellow amber	Acid, slight	−	+	−					
						Av.	439.1	5.38	1.42	4.29	7.37:1

[a] Glucuronic acid in 1-day collection.
Microscopic appearance of urine was normal for all dogs except that urine of dog No. 16 contained white blood cells and bile.

Blood Studies. Results of blood studies are shown in Table I. Blood studies were made at intervals of approximately 5, 8, and 13 months after start of the feeding test. Hemoglobin, red cell counts, white cell counts, and differential white cells shown in Table I are values obtained at 13 months or when the dogs were sacrificed. While there was a tendency toward a slight increase in red cells and white cell counts as the level of BHA in the diet was increased, the values are within the normal range. The feeding of BHA at the levels used here appeared to have no appreciable effect upon hemoglobin level or upon cell counts.

Urinalysis. Routine urinalyses were run on each dog several times during the feeding test. Values obtained at the end of the feeding period when the dogs were sacrificed are shown in Table II. Positive sugar tests were obtained on some of the dogs that received both the control diets and the diets containing BHA, although there were only two dogs in groups 1, 2, and 3 whose urine showed only a faint positive sugar test while urine from all four dogs in group 4 was strongly positive for sugar. Average glucuronate levels were increased in the urine from dogs receiving BHA in their diet.

Samples of urine collected from the dogs at time of sacrifice were assayed qualitatively for BHA, using a color reaction based on the reagent *N*-2,6-trichloro-*p*-benzoquinoneimine. It was observed that all of the dog urine samples reacted with the reagent to give shades of blue and green, indicating that the urine contained small amounts of ma-

terials that react similarly to BHA to produce colored products. Absorbance curves were plotted, and indicated that BHA or other compounds giving a similar color reaction were present in the urine of dogs receiving BHA in their diet at the levels fed.

Quantitative assays of urine for total sulfates and inorganic sulfates were run on composite samples consisting of three or more urine samples from three dogs in each lot. Results show that dogs on the control diet, and on a low level of BHA (5 mg. of BHA per kg. of body weight) had normal urinary sulfate ratios. Dogs on the intermediate level (50 mg. of BHA per kg. of body weight) had a slight increase in organic sulfate, while dogs fed the higher level of BHA (250 mg. of BHA per kg. of body weight) had a considerably increased proportion of organic sulfates in the urine.

General Appearance of Dogs. All of the dogs were alert and active, had healthy hair coats and mild dispositions, and appeared to be healthy and contented throughout the course of the feeding period. The feeding of BHA at the levels here used appeared to have no effect upon the outward appearance or general health of the dogs.

Autopsy Findings and Histological Studies. Gross examination of the organs at autopsy revealed one animal (dog No. 3 in control group) with focal endocardial calcification of the left atrium without other lesions. The remainder of the animals showed no gross lesions except for one dog in the group receiving the highest dosage (dog No. 16, group 4) which had bile in the urine. There was no obstruction of the common, cystic, or hepatic ducts in this dog, but the liver was bile stained and the capsular surface was finely granular.

Microscopic examination of representative sections from heart, liver, lung, pancreas, spleen, kidney, intestine, lymph node, stomach, thyroid, parathyroid, adrenal, gonad, and bone marrow revealed no changes beyond normal variation except in group 4 receiving the highest level of BHA in the diet. Abnormal variations in this group were all associated with the liver. Three of the four animals of group 4 had a liver cell degeneration and a diffuse granulocytic infiltration. The lobular structure of the liver was normal and there was no excessive connective tissue proliferation. There was diffuse parenchymal degeneration, with marked narrowing of the hepatic sinusoids. The Kupffer cells contained an increased amount of hemosiderin and there were focal accumulations of bile pigment in the periportal areas.

Functional and structural changes occurring during any hepatic disorder are usually more manifest in animals with insufficient dietary intake. The average daily feed intake of dogs 13, 14, and 15 in group 4 was lower than the average intake of dogs in other lots, but dog No. 15 with the lowest daily feed intake did not show signs of liver degeneration. However, during the course of the experiment it became apparent that this dog showed a definite dislike for feed containing the 250 mg. level of BHA. Because of this dog's dislike for BHA, it was decided to reduce the level of BHA in its feed, but to maintain as high a level as possible and yet allow proper feed intake for maintenance and growth. As a result, the average daily intake of BHA for dog No. 15 amounted to only 183 mg. of BHA per kg. of body weight, which may have accounted for the absence of liver degeneration in this dog.

Discussion and Conclusions

To all outward appearances, the dogs were not affected by the BHA in their diet except insofar as the BHA reduced palatability of the diet. There was a tendency toward reduced feed intake, particularly in group 4 receiving the highest level of BHA. Reduced feed intake was probably a contributing factor toward lower weight gains by the dogs in this group.

BHA in the concentrations here fed had no effect on the blood picture of the dog. Hemoglobin levels and cell counts did not seem to vary appreciably among the groups.

Results of urinalysis indicate increased levels of glucuronates in the dogs fed BHA, and a changed ratio of ethereal to inorganic sulfate. Positive sugar tests in urine from dogs fed BHA may have been due to the high levels of glucuronates in the urine. It would appear that the dog excretes BHA in the form of glucuronates and sulfate conjugates. Similar BHA excretion pathways have been reported for the rabbit (*1*).

At the time the dogs were sacrificed, they all appeared vigorous and well nourished, and of the treated animals, only the one animal in the group receiving the highest dosage of BHA appeared to have any gross pathological condition.

Liver injury was found by microscopic examination in three of the four animals in the group receiving the highest dosage of BHA. All other organs and tissues examined appeared to be normal.

It would appear from these studies that the dog is able to ingest BHA at the two levels here fed (5.0 mg. and 50.0 mg. per kg. of body weight per day) with no deleterious effects. However, when the dog is fed 250 mg. of BHA per kg. of body weight per day, the dog is unable to cope with this large amount without damage to the liver. Because three of the four dogs in group 4 weighed over 6000 grams at the end of the feeding period, these three dogs were each receiving more than 1.5 grams of BHA per day. The other dog in group 4, dog No. 15, did not consume as much food per day, did not gain as much weight, and refused to consume BHA at a level of 250 mg. per kg. of body weight. It did consume an average of 183 mg. of BHA per kg. per day, so that at the end of the test period this dog was consuming approximately 786 mg. of BHA per day, or roughly half of the amount consumed by the other three dogs in the group. This dog showed no sign of liver injury at the time of sacrifice.

Present regulations (*3*) allow the use of BHA in lard at a level not to exceed 0.01%. The lowest level of BHA fed here to dogs was 5 mg. per kg. of body weight per day, which amounted to approximately 0.22% of BHA in the lard of the diet or 22 times the maximum allowed in lard for human consumption. Group 3 received approximately 2.24% of BHA in the lard, or 220 times the maximum amount allowed in lard. At 22 and 220 times the maximum allowable levels of BHA in the lard, no evidence of injury was observed. It was only in group 4 where three of the dogs received 250 mg. of BHA per kg. of body weight per day, or approximately 11.0% of BHA in the lard of the diet and 1100 times the maximum level allowed in lard, that any liver injury was observed. The one dog in group 4 (dog No. 15) that actually consumed about half of this amount, or approximately 550 times the level allowed by Meat Inspection Division, Bureau of Animal Industry regulations, did not show liver injury.

Acknowledgment

The authors are grateful to B. H. Spargo, Department of Pathology, University of Chicago Medical School, for his counsel and help in making the microscopic examination of tissues, and in the interpretation of the findings.

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Tests on Mice for Evaluating Carcinogenicity

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In a report of the Food Protection Committee (1959) a "practical procedure for evaluating carcinogenicity" is described which includes (a) continuous feeding of various levels of the test substance to rats and mice for their normal lifespans and to dogs for at least four years, (b) repeated topical application of various amounts to the skin of mice for the normal lifespan, and (c) a single subcutaneous injection in mice of not more than 100 mg of the test substance with observation of the animals for the rest of their lives. No substance is known to us on which all this information has been collected.

Having conducted, however, two-year rat-feeding studies (a longer period than the median lifespan) on several substances and having in addition some data on one-year dog-feeding studies (considerably less prolonged than the four-year period recommended), we selected compounds that had shown no evidence of carcinogenicity as well as substances judged to be carcinogenic in at least one species to see whether the treatment of mice topically or subcutaneously would furnish additional evidence of carcinogenic potency or lack of it. These tests on mice offered several possible advantages, e.g., confirmatory evidence in an additional species by other routes, species differences in sensitivity, a more rapid response, or less expense. Six substances were ultimately selected (Fig. 1) based on our data plus some from other laboratories as indicated. The three carcinogens are aminotriazole, Aramite,[2] and Flectol H;[3] the three non-carcinogens are butylated hydroxyanisole (BHA), dioctyl adipate, and methoxychlor.

To give the evidence on which the judgments of carcinogenicity were based, a brief summary will be presented of the incidence of tumors of various types in the rats and dogs that had been maintained for prolonged periods on diets containing these six substances.

Aminotriazole.[4] Rats were maintained for two years on diets containing 0, 10, 50, or 100 ppm of aminotriazole.

[1] Deceased.

[2] Trademark of the Naugatuck Chemical Division of the U. S. Rubber Co., Naugatuck, Connecticut, for the miticide chemical 2-(*p-tert*-butylphenoxy)isopropyl 2-chloroethyl sulfite.

[3] Trademark of the Monsanto Co., St. Louis, Missouri. Flectol H is a polymer of 1,2-dihydro-2,2,4-trimethylquinoline, which is used industrially as a rubber antioxidant.

[4] These experiments were conducted by Hazleton Laboratories, Inc., Falls Church, Virginia, and are unpublished. The data were made available through the courtesy of Dr. C. B. Shaffer, American Cyanamid Co., Wayne, New Jersey.

Rats on the 100-ppm dietary level showed a high incidence of thyroid adenoma (15 in 27 rats examined); the incidence was lower (1-3 in 27 rats) in the groups given less aminotriazole. Mammary and other tumors were distributed in a random fashion. Whether the thyroid adenoma ultimately becomes malignant has been debated extensively by pathologists; at least one of the several groups of pathologists examining these sections believed the changes to be malignant.

Dogs (2-4 per group) were fed for a period of one year diets containing 0, 10, 50,

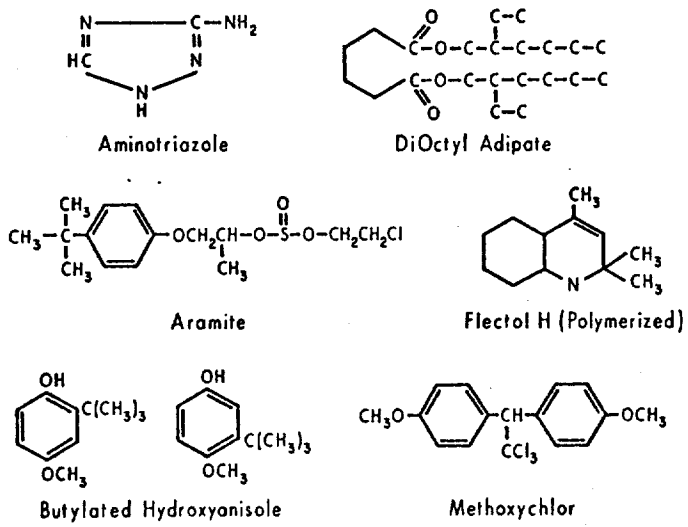

FIG. 1. Structural formulas of compounds used in these studies.

100, or 500 ppm of aminotriazole. No tumors were detected, in the thyroids or elsewhere.

Aramite. Data were taken from the work of Oser and Oser (1960, 1962). In the first two-year study, the diets contained 0, 500, 1580, or 5000 ppm of Aramite. In the livers of the 500-ppm group, one example of hyperplastic change was described. At the mid-level, changes in 2 of 21 livers were described as malignant. In the 5000-ppm group, 6 of 20 livers exhibited hepatomata or cholangiomata. In a second study, three strains of rats were maintained on diets containing 0, 100, 200, or 400 ppm of Aramite for two years. Hyperplastic nodules were found in the livers at each level in one or more of the strains; the incidence increased with increasing dose.

Bile duct adenomata were found in two of the three strains of rats. "Occasional mammary or uterine tumors" were also found (Popper *et al.*, 1960).

Groups of dogs given 0, 500, or 1580 ppm of Aramite for a year developed no tumors. In other dogs whose diets contained 0, 500, or 840-1429 ppm of Aramite for periods up to three and one-half years, tumors were found. Of the seven dogs examined at 500 ppm, two had liver bile duct cancer, three had cancer of the gall bladder, and six had cancer of the gall ducts. At 840-1429 ppm, two of the 12 examined had bile duct cancer; furthermore, in the extrahepatic biliary system, six had cancer of the gall bladder, and six had cancer of the ducts (Steinberg *et al.*, 1960).

Butylated hydroxyanisole. Three long-term rat feeding studies have been reported. In one study, groups of rats were given for one year diets containing 0, 0.00135,

or 0.00675% of BHA. Two fibroadenomata were noted in 18 female rats on one of 4 diets at the higher level (Graham *et al.*, 1954). Other groups of rats were given diets containing 0, 0.003, 0.03, 0.06, or 0.12% for 21-22 months. No pathologic changes were attributed to the treatment. Tumors were not reported (Wilder and Kraybill, 1948). In the third study, rats were given diets containing 0, 0.01, or 0.1% for two years; tumor incidence was not reported (Brown *et al.*, 1959). There seems little likelihood that BHA is a carcinogen.

Dogs were given 0, 0.0176, or 0.176% of BHA for 15 months; no tumors were found (Wilder *et al.*, 1960). In another study, dogs were given 0, 0.001, 0.01, 0.1, or 0.3% for one year. One tumor was reported, namely, an adenocarcinoma in a control dog (Hodge *et al.*, 1964).

Dioctyl adipate. In our laboratory (unpublished data) dioctyl adipate was fed to rats for two years, using diets containing 0, 0.1, 0.5, or 2.5%. A total of 33 tumors was found. A number of these were lymphomata and adenomata, and there was one fibroma, two carcinomata of the breast, and one carcinoma of the kidney. The incidence of these tumors was not related to the dietary treatment.

Dogs were maintained for one year on diets containing 0, 0.07, 0.15, or 0.2% of dioctyl adipate. No tumors were found.

Flectol H. Rats were maintained in this laboratory for two years on diets containing 0, 0.01, 0.1, or 1.5% of Flectol H (unpublished data). Two forms of tumor, lymphoma and adenoma, were the most numerous. The incidence of lymphoma (number of animals with lymphoma divided by the total number examined) tended to increase with increasing dietary percentages. Lymphoma incidences for the controls (0%) and for rats fed dietary levels of 0.01, 0.1, and 1.5% were 1/9, 1/10, 3/15, and 8/8, respectively. The incidences of adenomata for control rats and for rats from the Flectol H diets were 3/9 (0%), 4/10 (0.01%), 12/15 (0.10%), and 3/8 (1.5%). Cholangiofibrosis, without metastases, was observed in 3 rats fed the highest (1.5%) dietary level (Panner and Packer, 1961). A few fibromata and other tumors were noted, but the incidence of these (total of four) was limited to four different rats and was not related to dose.

No tumors were found in dogs (3 per group) maintained for one year on diets containing 0, 0.008, 0.03, or 0.15% of Flectol H.

Methoxychlor. Methoxychlor was fed to rats for a two-year period in the following dietary concentrations: 0, 0.0025, 0.02, or 0.16% (Hodge *et al.*, 1952). Several tumors were found, specifically a neurofibroma in a rat given the 0.0025% diet, a mammary tumor in a rat given the 0.02% diet, and a mammary tumor, an ovarian cyst, an adenoma of the pancreas, an epidermoid carcinoma, and a tumor of the abdominal wall in rats given the 0.16% diet. Such tumors appear spontaneously from time to time with varying frequencies in control rats of our colony (Crane, 1958).

Dogs were given diets containing 0, 0.06, 0.3, or 1.0% of methoxychlor for one year. No tumors were discovered.

METHODS

Animals. Male and female mice of the C3H/Anf strain[5] were housed in plastic cages on wood shavings in groups of 12 or 13 to a cage, and supplied ad libitum with

[5] Obtained from Cumberland View Farms, Clinton, Tennessee.

commercial laboratory chow pellets[6] and tap water. Each treatment group of 100 mice was 2-4 months old at the time of the first treatment.

Materials. The materials tested were aminotriazole,[7] Aramite,[8] butylated hydroxyanisole,[9] dioctyl adipate,[10] Flectol H,[11] and methoxychlor.[12]

The vehicle used for the subcutaneous injection of the forementioned materials was trioctanoin.[13] All, except aminotriazole, were readily soluble (50 mg/ml) in trioctanoin. The aminotriazole mixture was placed in a Waring blendor for 15 minutes and a suspension was prepared that could be injected. All the materials, except aminotriazole, were prepared for high-dose skin application as 5% solutions in acetone.[14] The aminotriazole (5.0 g) was first dissolved in 35 ml of anhydrous methanol,[15] and a quantity of acetone was added to bring the volume of this solution to 100 ml. The 0.05% solutions for the low-dose skin applications were prepared by taking a 1-ml aliquot of the 5.0% solution and diluting to 100 ml.

Treatment. Single subcutaneous injections (0.02 ml) were made with a 27-gauge needle under the skin of the mouse in the mid-scapular region. The injected material formed a small mass or nodule which was gently massaged with the finger tips until the nodule was no longer palpable. The mouse was then returned to its cage. Fifty male and fifty female mice were used in each treatment group.

For the skin studies, each mouse was shaved with an electric clipper, and the hair was removed from an area of approximately 3 × 3 cm in the scapular region. A volume of 0.20 ml of the acetone solution was applied to this area once each week. Immediately after treatment the mouse was isolated in a 250-ml beaker until the skin appeared dry; then the mouse was returned to its cage. Fifty male and fifty female mice were used in each treatment group.

All mice were examined each week and observations of the skin condition were recorded. Weekly weight of each cage group was recorded. Mouse cages, injection equipment, and the solutions carried a color-code that was assigned initially to each treatment group. The mice were observed until death, and the carcass was placed in a bottle containing 20% formalin. The mouse was later submitted to gross autopsy examination, and, if autolysis had not occurred, sections of the skin were taken for histologic study.

[6] Purina Laboratory Chow Pellets, Ralston Purina Co., St. Louis, Missouri.

[7] This sample was an "analytical grade" supplied by the Agricultural Division, American Cyanamid Co., Princeton, New Jersey.

[8] Supplied by the Naugatuck Chemical Division, U.S. Rubber Co., Naugatuck, Connecticut.

[9] Supplied by Eastman Chemical Products, Inc., a subsidiary of Eastman Kodak Co., Kingsport, Tennessee.

[10] Supplied by Monsanto Co., St. Louis, Missouri. The sample was 97% pure.

[11] Supplied by Dewey and Almy Chemical Division, W. R. Grace & Co., Cambridge, Massachusetts.

[12] This was a recrystallized sample from E. I. du Pont de Nemours & Co., Wilmington, Delaware.

[13] Eastman Organic Chemicals Department, Distillation Products Industries, Rochester, New York.

[14] "Baker Analyzed" Reagent, J. T. Baker Chemical Co., Phillipsburg, New Jersey.

[15] Analytical Reagent grade, Mallinckrodt Chemical Works, St. Louis, Missouri.

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Test substance	Sex
Control	M
Aminotriazole	M
Aramite	M
BHA	M
Dioctyl adipate	M
Flectol H	M
Methoxychlor	M
Control	F
Aminotriazole	F
Aramite	F
BHA	F
Dioctyl adipate	F
Flectol H	F
Methoxychlor	F

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RESULTS

Single Subcutaneous Injections

No effect on the body weight of the mice was noted in any of the injected groups. During the first 15 weeks the average body weights for the various treated groups and for the control mice increased by 4-6 g (Table 1).

The median survival time for each cage group of mice (4 cages, 50 mice) was determined, and the mean and the standard error were determined for each treatment group (Table 2). The longest survival (575 days) for male mice was recorded for the control (trioctanoin-injected) animals. Average median survival values in the treated groups ranged from 273 to 533 days. The mean median survival time for the control female mice was 337 days (SE ± 20 days), and the average values for the treated groups were all somewhat greater, ranging from 351 to 444 days. Evidence can be marshalled to indicate that these deaths were not related to the treatment.

TABLE 1
MEAN BODY WEIGHT OF MICE GIVEN A SINGLE SUBCUTANEOUS INJECTION OF 10 MG OF TEST SUBSTANCE

Test substance	Sex	Initial weight (g)				15 Weeks after injection (g)			
		Group 1	Group 2	Group 3	Group 4	Group 1	Group 2	Group 3	Group 4
Control	M	25	24	27	25	30	31	33	29
Aminotriazole	M	28	27	27	26	30	28	31	27
Aramite	M	25	25	26	26	30	30	26	26
BHA	M	24	23	26	26	28	29	29	27
Dioctyl adipate	M	25	23	26	26	30	27	29	29
Flectol H	M	25	24	27	26	30	28	32	30
Methoxychlor	M	23	24	26	26	27	28	29	29
Control	F	21	21	23	22	25	25	29	28
Aminotriazole	F	24	23	22	23	26	27	26	29
Aramite	F	22	21	23	22	26	26	27	26
BHA	F	21	21	23	22	26	25	29	27
Dioctyl adipate	F	20	21	22	22	24	27	26	27
Flectol H	F	18	21	23	22	25	26	27	27
Methoxychlor	F	21	25	23	22	26	29	28	25

Throughout the experiment it was noted that several (2-6) deaths would occur on a single day. For example, among the female control mice 10 deaths were recorded from one cage on the 378th day, and 4 deaths were recorded from a second group (control) of 13 mice on the 379th day. Six male mice from one cage group (treated with dioctyl adipate) were found dead on the 146th day after injection, while in another group 5 were found dead on the 139th day. Multiple deaths had been observed earlier in mice treated with methoxychlor; a total of 6 mice died in one cage group on the 13th (4 mice) and 14th days (2 mice) after injection. In the total of 28 cages housing the 350 male mice, there were only 5 cages in which two or more deaths did not occur on a single day at least once during the two-year period. Among the female mice, multiple deaths occurred in 27 of the 28 cages. Gross autopsy examinations revealed, in many instances, signs of pneumonitis frequently bilateral in ex-

tent. It thus appears that the deaths in many cases probably resulted from intercurrent disease and were not direct toxic responses.

Gross weekly examinations of the mice for as long as they survived did not reveal any tumors at the site of injection. One breast tumor (fibromyxoma) was observed at the time of autopsy in a male mouse that had received a subcutaneous injection of dioctyl adipate nearly 14 months earlier. During the first year after injection, no tissue sections were taken when the gross pathologic findings were negative. Thereafter, sections of the skin and subcutaneous area were taken for histologic study if autolysis had not occurred.

TABLE 2

MEANS AND STANDARD ERRORS OF MEDIAN SURVIVAL TIMES OF MICE GIVEN A SINGLE SUBCUTANEOUS INJECTION OF 10 MG OF TEST SUBSTANCE

Test substance	Males (days)			Females (days)		
	Mean[a]	SE	Range	Mean[a]	SE	Range
Controls	575	8	549-581	337	20	275-278
Aminotriazole	438	83	163-570	351	59	182-489
Aramite	533	46	435-625	401	32	290-448
BHA	508	51	411-667	408	52	251-525
Dioctyl adipate	273	66	141-435	355	46	249-498
Flectol H	475	43	380-602	444	26	376-518
Methoxychlor	372	112	14-564	419	25	365-500

[a] Mean of four cage groups.

TABLE 3

NUMBERS OF MICE FROM WHICH TISSUES WERE TAKEN FOR HISTOLOGIC STUDY AFTER A SINGLE SUBCUTANEOUS INJECTION OF 10 MG OF TEST SUBSTANCE

Test substance	Male	Female	Total
Control	14	1	15
Aminotriazole	19	14	33
Aramite	22	5	27
BHA	29	13	42
Dioctyl adipate	14	12	26
Flectol H	14	9	23
Methoxychlor	17	7	24

Tissues from a total of 140 mice were examined microscopically. In only 3 groups were less than 9 mice available, and in 9 groups there were 14 or more (Table 3). The skin sections of many mice were essentially normal. Occasionally, thin fibrous-walled cysts were found at the site of injection (Figs. 2-4). Some of the cysts appeared empty while others had clusters of foamy macrophages associated with them. The cysts appeared to be smaller in size as the time after injection increased; in fact, cysts or their remnants were not detected grossly in mice that died late in the experiment. Ectoparasites on the hair or skin surface were seen frequently, and liver parasites were noted in a few mice.

In addition, tissues from 4 mice injected with trioctanoin and 6 mice injected with Aramite (10 mg/mouse) in trioctanoin were obtained at 1 and 4 months after in-

FIG. 2. Subcutan
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TIMES OF MICE GIVEN A SINGLE
OF TEST SUBSTANCE

	Females (days)		
e	Mean[a]	SE	Range
31	337	20	275-278
70	351	59	182-489
35	401	32	290-448
37	408	52	251-525
35	355	46	249-498
2	444	26	376-518
4	419	25	365-500

FOR OLOGIC STUDY AFTER A SINGLE
E TEST SUBSTANCE

Female	Total
1	15
14	33
5	27
13	42
12	26
9	23
7	24

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FIG. 2. Subcutaneous cyst in a control, female mouse given a single subcutaneous injection of 0.02 ml of trioctanoin. The animal died 29 weeks after the injection.

FIG. 3. Subcutaneous cyst in a female mouse given a single subcutaneous injection of 10 mg of Aramite in 0.02 ml of trioctanoin. The animal died 4 weeks after the injection.

FIG. 4. Subcutaneous cyst in a male mouse given a single subcutaneous injection of 10 mg of Aramite in 0.02 ml of trioctanoin. The animal died 55 weeks after the injection.

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Skin Application—L

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Median survival ti

MEANS AND STAN

Rank	Male Test substance
1	Aminotriazole
2	Aramite
3	Controls
4	Methoxychlor
5	Dioctyl adipate
6	Flectol H
7	BHA

[a] Days after start of
[b] Mean of four cage g

mice of each sex per
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Male mice tended to
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jection. Both treatment groups showed signs of acute and chronic inflammation in the subcutaneous tissue. The formation of single or multiple cysts with thin fibrous walls, foreign body reactions, and phagocytosis of a dull-brown pigment were noted in the trioctanoin- and Aramite-treated groups. No evidence of neoplasia was found.

Skin Application—Low Doses

Seven groups of mice (50 males and 50 females per group) were given weekly applications of 0.20 ml of acetone (controls) or of 0.10 mg of each test compound in 0.20 ml of acetone. The average body weights of each cage group were recorded weekly; no differences were observed between the control and treated groups.

Median survival times (days) were determined for each cage group (4 cages of 50

TABLE 4
MEANS AND STANDARD ERRORS OF MEDIAN SURVIVAL TIMES OF MICE GIVEN WEEKLY SKIN APPLICATIONS OF 0.1 MG OF TEST SUBSTANCE

	Males (days)[a]			Females (days)		
Rank	Test substance	Mean[b]	SE	Test substance	Mean[b]	SE
1	Aminotriazole	374	65	Dioctyl adipate	310	55
2	Aramite	426	83	BHA	323	56
3	Controls	430	57	Aramite	328	36
4	Methoxychlor	434	32	Aminotriazole	334	50
5	Dioctyl adipate	447	19	Methoxychlor	342	36
6	Flectol H	485	19	Flectol H	385	52
7	BHA	519	29	Controls	393	27

[a] Days after start of treatment. To convert to days of age, add 191.
[b] Mean of four cage groups.

mice of each sex per treatment group), and the mean and standard error of the mean were determined. These values, ranked in the order of increasing survival time, are given in Table 4. The median survival times show no characteristic pattern and the values for most of the treated groups lie within the limits of the control values. Male mice tended to outlive female mice. There were a number of instances where several mice from one cage group were found dead in a single day; for example, among the female mice treated with BHA, deaths of 4, 5, and 8 mice occurred on one day in three of the four cage groups. Among female mice treated with dioctyl adipate, multiple deaths (6 or 7 mice) were also noted in three of the four cage groups. In two instances, multiple deaths in mice treated with BHA or dioctyl adipate occurred within 7 or 8 days of each other. The pattern suggests that an infection (probably pulmonary) was responsible.

The total dose administered to these mice varied considerably because their survival times varied widely. The data for total doses are summarized in Table 5. The control data give the lowest and highest total doses of acetone applied to the skin. Among the male mice the first mice to die had received total doses ranging from 0.8 to 2.7 mg and the last mice to die had received total doses ranging from 8.7 to 10.4 mg. In female mice comparable figures range from 0.1 to 2.5 mg (first mice to die) to 7.4 to 9.8 mg for the last mice to die.

No gross changes of the skin were observed in any of the mice. Skin sections from a total of 205 mice were examined microscopically. The number and sex of these mice are given in Table 6. There were no histologic alterations observed in the sections of the skin that could be attributed to the treatment. Two skin sections taken from mice given repeated doses of BHA showed areas of ulceration that were healing. Presumably, these ulcerations resulted from injury during the clipping of the hair prior to application of the test material. Focal hepatic necrosis was observed in the livers of two female mice (both from the same cage group) that had received weekly

TABLE 5
TOTAL DOSES ADMINISTERED TO MICE BY WEEKLY SKIN APPLICATIONS OF 0.1 MG OF TEST SUBSTANCE

Test substance	Males (mg/mouse)		Females (mg/mouse)	
	Lowest	Highest	Lowest	Highest
Control	2.4[a]	21.0[a]	2.8[a]	17.2[a]
Aminotriazole	1.9	9.5	1.2	9.0
Aramite	1.2	9.0	0.3	6.9
BHA	2.7	10.4	0.2	8.3
Dioctyl adipate	2.4	8.8	2.5	9.8
Flectol H	3.2	8.7	1.8	8.9
Methoxychlor	0.8	10.4	0.1	7.4

[a] Total milliliters of acetone per mouse.

TABLE 6
NUMBERS OF MICE FROM WHICH SKIN SECTIONS WERE TAKEN FOR HISTOLOGIC STUDY AFTER WEEKLY SKIN APPLICATIONS OF TEST SUBSTANCE

Test substance	Low-dose study[a]			High-dose study[b]		
	Males	Females	Total	Males	Females	Total
Controls	14	1	15	18	12	30
Aminotriazole	20	20	40	20	13	33
Aramite	15	24	39	10	5	15
BHA	15	15	30	21	13	34
Dioctyl adipate	13	13	26	19	13	32
Flectol H	11	16	27	13	9	22
Methoxychlor	13	15	28	15	8	23
Total	101	104	205	116	73	189

[a] Dose was 0.1 mg/mouse, applied weekly.
[b] Dose was 10 mg/mouse, applied weekly.

applications of 0.10 mg of Flectol H. One male mouse treated with Aramite showed central fatty change of the liver, and one female mouse given weekly doses of BHA had an ovarian cyst.

Skin Application—High Doses

A total of seven groups of mice received weekly skin applications of 0.20 ml of acetone (controls) or of 10 mg of the test material (except aminotriazole) in acetone solution (50 mg/ml). The aminotriazole was administered at the same dose and volume in 35:65 methanol-acetone.

EV

Average body weight
as those of the control
were noted over the fir
Mean values for the
each sex and their stan
ascending order of surv
all treated male groups
control mice. Female mi

MEANS AND STANDARD

Rank	Test substance	Males (
1	Controls	
2	Aminotriazole	
3	Flectol H	
4	BHA	
5	Dioctyl adipate	
6	Aramite	
7	Methoxychlor	

[a] Days after start of trea
[b] Mean of four cage grou

TOTAL DOSES ADMINISTERED

Test substance	I
Controls	
Aminotriazole	
Aramite	
BHA	2
Dioctyl adipate	
Flectol H	
Methoxychlor	1

[a] Total milliliters of acetor

that were not significantl
mice.
On a number of occasi
day. The number of dead
related to treatment or t
The total doses admini
values are given in Table
(a single application) to
(10-20 mg) occurred in t
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any of the mice. Skin sections from
ally. The number and sex of these
logic alterations observed in the sec-
treatment. Two skin sections taken
s of ulceration that were healing.
ury during the clipping of the hair
epatic necrosis was observed in the
ge group) that had received weekly

EEKLY SKIN APPLICATIONS OF
TANCE

Females (mg/mouse)	
Lowest	Highest
2.8[a]	17.2[a]
1.2	9.0
0.3	6.9
0.2	8.3
2.5	9.8
1.8	8.9
0.1	7.4

TAKEN FOR HISTOLOGIC STUDY AFTER
EST SUBSTANCE

High-dose study[b]		
Males	Females	Total
18	12	30
20	13	33
10	5	15
21	13	34
19	13	32
13	9	22
15	8	23
116	73	189

treated with Aramite showed
e given weekly doses of BHA

pplications of 0.20 ml of ace-
ot aminotriazole) in acetone
d at the same dose and vol-

Average body weights, recorded weekly, of all test groups were essentially the same as those of the control mice. Gains in the average body weights ranging from 1 to 3 g were noted over the first 15 weeks of the study.

Mean values for the median survival times (days) from the four cage groups of each sex and their standard errors are given in Table 7. These values are ranked in ascending order of survival time from the first day of treatment. Survival times for all treated male groups were equal to or greater than that observed among the male control mice. Female mice treated with various test agents had average survival times

TABLE 7

MEANS AND STANDARD ERRORS OF MEDIAN SURVIVAL TIMES OF MICE GIVEN WEEKLY SKIN APPLICATIONS OF 10 MG OF TEST SUBSTANCE

	Males (days)[a]			Females (days)		
Rank	Test substance	Mean[b]	SE	Test substance	Mean[b]	SE
1	Controls	386	9	Aminotriazole	309	39
2	Aminotriazole	402	54	Controls	313	23
3	Flectol H	439	22	Flectol H	330	34
4	BHA	441	56	BHA	356	45
5	Dioctyl adipate	445	30	Dioctyl adipate	417	23
6	Aramite	452	22	Aramite	441	20
7	Methoxychlor	459	22	Methoxychlor	452	19

[a] Days after start of treatment. To convert to days of age, add 168.
[b] Mean of four cage groups.

TABLE 8

TOTAL DOSES ADMINISTERED TO MICE BY WEEKLY SKIN APPLICATIONS OF 10 MG OF TEST SUBSTANCE

Test substance	Males (mg/mouse)		Females (mg/mouse)	
	Lowest	Highest	Lowest	Highest
Controls	0.20[a]	22.0[a]	3.8[a]	17.4[a]
Aminotriazole	50	1100	80	920
Aramite	20	1040	120	950
BHA	270	1110	250	840
Dioctyl adipate	30	920	190	1010
Flectol H	10	890	180	810
Methoxychlor	110	980	10	880

[a] Total milliliters of acetone per mouse.

that were not significantly shorter than the mean survival time for the control female mice.

On a number of occasions in this study, multiple deaths were observed on a single day. The number of dead varied from 4 to 7. These occurrences did not appear to be related to treatment or to the number of applications.

The total doses administered to the mice showed considerable variation, and the values are given in Table 8. The total amounts applied to the mice varied from 10 mg (a single application) to 1100 mg for the male or female mice. The very low values (10-20 mg) occurred in the groups where the first mouse died within a week or two after the start of the study. The maximal total doses ranged from 890 to 1110 mg for

male mice and from 810 to 1010 mg for female mice. Substantial total amounts of the vehicle (acetone) were applied to the control mice; the highest cumulative doses were 17 and 22 ml, respectively, for the last female and male survivors. These amounts are equivalent in some cases to slightly more than half the body weight of the mice.

No gross evidence of tumor formation was detected in the skin of the control or of the treated mice (Figs. 5 and 6). Occasionally, hair loss was noted in control or treated groups. Skin sections examined from a total of 189 mice (Table 6) showed no evidence of benign or malignant tumors. At gross autopsy examination, the liver of a male mouse that had received a total of 43 doses (10 mg/dose) of Flectol H

FIG. 5. Normal skin from a female mouse given 47 applications (10 mg each) of Aramite.

showed yellow nodules. The microscopic sections of this liver revealed a nodule of well differentiated liver cords, without evidence of pleomorphism. This tumor was considered to be an isolated spontaneous benign tumor or hamartoma. Other findings were frequent ectoparasites of the skin surface or in the hair, variable numbers of mast cells in the dermis and subcutis, and the occurrence of liver worms in a few animals.

DISCUSSION

The median survival times showed no characteristic pattern; i.e., none of the six chemicals had any adverse effect on survival. In the low-dose study, females tended to outlive the males; in the high-dose study, the reverse was true for some of the groups, but no difference could be shown in others. Median survival times ranged from over 300 to nearly 600 days for the mice on the low dose, and from over 450 to over 600 days for mice on the high dose.

In the Food Protection Committee's report (1959), the mouse is named as "the

species of choice for
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that of the rat." The
the skin of the mouse.
Thus, there is no indi
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FIG. 6. Ulcerated sk

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Six chemical compou
cutaneous implantation
[2-(*p-tert*-butylphenoxy
2,2,4-trimethylquinoline)
hydroxyanisole, dioctyl
chronically to rats and d
Six groups of 100 mice

species of choice for tests using this route (skin) of administration" because "the skin epithelium of the mouse is generally more responsive to tumor formation than that of the rat." The literature is rich in reports of tests of potent carcinogens on the skin of the mouse. In this study, no tumors were found at the site of treatment. Thus, there is no indication that for these not-potent carcinogens the proposed program of subcutaneous or dermal applications to mice would have added useful information or permitted a shorter study, e.g., one of 6 months' duration.

FIG. 6. Ulcerated skin of a male mouse given 41 applications (10 mg each) of Aramite.

Some histologic changes described as cysts were seen in the mice given subcutaneous injections. These cysts were thin-walled, with some evidence of compression, and filled with a clear fluid. Almost no indication of inflammation or irritation was found in the adjacent tissues. Even in a cyst that had persisted for many months, no sign of adjacent tissue reaction was detected.

The dermal applications gave only normal-appearing epidermis. In a number of mice, signs of healing ulcerations probably represented sequelae of accidental nicks inflicted during the weekly clipping of the hair. No evidence of epithelial proliferation appeared.

SUMMARY

Six chemical compounds were tested for carcinogenic potency in mice (C3H/Anf) by subcutaneous implantation or repeated skin application. Three compounds, aminotriazole, Aramite [2-(*p-tert*-butylphenoxy)isopropyl 2-chloroethyl sulfite], and Flectol H (a polymer of 1,2-dihydro-2,2,4-trimethylquinoline), were carcinogens or suspected carcinogens. Three compounds, butylated hydroxyanisole, dioctyl adipate, and methoxychlor had shown no carcinogenic potential when fed chronically to rats and dogs.

Six groups of 100 mice (50 of each sex) were given single, subcutaneous injections (10 mg/mouse)

of the test compounds. A seventh group of 100 mice was given the injection vehicle only (triocta-noin) and served as controls. No evidence of carcinogenic activity attributable to the treatment was noted over periods of observation averaging 273-575 days. Six groups of 100 mice (50 of each sex) were given weekly skin applications of 0.1 mg of the test compound in acetone, and six additional groups of 100 mice were given weekly applications of 10 mg of the test compound in acetone. A total of 200 control mice (100 of each sex) were given weekly applications of acetone only (0.20 ml). No gross evidence of tumor formation was detected in the skin of these mice (mean survival times ranged from 310 to 519 days for the low dose and from 309 to 459 days for the high dose). Microscopic examination of the skin from 205 mice from the low-dose study and of 189 mice from the high-dose study showed no evidence of tumors.

Test procedures on mice observed for the remainder of their lives and involving weekly applications to the skin or single subcutaneous implantations of chemical agents having low carcinogenic potential are not adequate substitutes for tests by other routes of administration. For the compounds selected, these procedures failed to contribute significant additional information.

ACKNOWLEDGMENT

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

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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

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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*

Strain	No. of animals*	Test substance	Dose IU	Dose mg/kg	Dose regime
		MICE			
Evans No. 1	10	BHT		750	Daily administration for 32–46 days prior to mating and up to day 18 of pregnancy
Evans No. 1	83	BHT		1000	Single administration on a specific day of pregnancy (day 1–18)
Evans No. 1	10	BHT		750	Daily administration on days 1–18 of pregnancy
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			
Tuck albino	32	BHA		750	Daily administration on days 1–20 of pregnancy†
		BHT		750	
		Vitamin A	5000		
Tuck albino Benger hooded	47	BHA		750	Daily administration for 70 days before pairing, continuing through pregnancy
		BHT		750	
		Vitamin A	5000		
Tuck albino Carworth SPF albino Benger hooded	111	BHA		1000	Single administration on day 9, 11 or 13 of pregnancy
		BHT		1000	
		Vitamin A	60,000		
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 pre-mating period calculations were made of a Mating Index (no. of matings/no. of opportunities to mate $\times 100$, 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 $\times 100$). 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

daily 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 Benger 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 treat-ment	No. in group	No. preg-nant	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* (60,000 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*

				Type of abnormality																
				Gross examination			Skeletal examination			Sternal ossification centres			Soft tissue examination							
Test substance	Day of treatment	Proportion of rats with abnormal foetuses	Total no. of abnormal foetuses	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.

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.

Vitamin A	13	6/6	14			1
Arachis oil	13	0/6	0			
None	—	1/14	1			

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

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.

‖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*

		Resorptions, as percentage of total litter				
Species	Strain	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 μg/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 early 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 traités 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 auf.

C

Effect of Antioxidants in Feed Fats (butyloxyanisole and butyloxytoluene) on Some Aspects of Fat Metabolism in Animals

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The preservation of fat and of fat-containing products from oxidation, in addition to having a great economic significance, also represents a hygienic problem, to the extent that oxidation and polymerization products of fats manifest a harmful effect on the organism (toxic, cocarcinogenic, and carcinogenic). For this reason, both the physical, as well as the chemical methods of preserving fats from oxidation deserve attention.

Inasmuch as the oxidation of fat bears a chain character, the reaction may be considerably slowed down by means of oxidation inhibitors. Butyloxyanisole, butyloxytoluene and gallic acid esters represent the most effective and promising of the synthetic antioxidants for utilization in the U.S.S.R.

Due to the fact that antioxidants represent chemical substances that are foreign to the organism, in order to be utilized in the food industry, there may be allowed only absolutely harmless oxidation inhibitors, which do not change the organoleptic properties of the products.

On the basis of papers presented in preceding years, at the Department of Nutritional Hygiene of the Central Institute for the Advanced Training of Doctors (1 - 3), butyloxyanisole and butyloxytoluene have been acknowledged as comparatively low-toxic substances. Small amounts of them (0.01 - 0.02% relative to the fat in the diet), within the framework of the investigations that had been carried out, proved to be harmless for the organism.

However, in the problem of studying any possible harmful effect of synthetic antioxidants on the organism, there still remain a number of interesting, but up to the present time insufficiently studied, problems, including the metabolism, in the organism, of fat, which contains antioxidants.

In the literature there exist indications that antioxidants, appar-

ently, exert an influence on the processes of fat metabolism. In the experiments of Johnson and his coauthors (4), who had fed to rats butyloxyanisole and butyloxytoluene, it has been noted that the utilization of these antioxidants, in amounts of 0.1 and 0.5% relative to the diet, increases the stability of perinophric fat in rats. Johnson and Hewgill (5) have shown that feeding the animals butyloxyanisole and particularly, butyloxytoluene, in concentrations of 0.3 - 0.5%, leads to an increase in the cholesterol content in the blood serum. Small doses of butyloxytoluene (0.001%) did not produce such changes. Day and his coauthors (6) reported on the possible interrelationship between the metabolism of butyloxytoluene (in relatively large dosages), fatty acids and the transport of cholesterol and phospholipids. The abovementioned researchers carried out experiments, basically, with rather large doses of antioxidants (aggravation by 100 - 500 times). There are no domestic (Soviet) papers on the effect of phenol antioxidants on fat metabolism.

We made an attempt to follow up on how the feeding of fat with butyloxyanisole and butyloxytoluene exerts an influence on some aspects of fat metabolism.

Experiments were conducted on male rats with an initial weight of 120 - 150 grams that were given, in the course of 30 - 35 days, the following diet: 15 grams of food concentrates (containing scoured wheat flour, scoured oat flour, millet flour, dry wheat germ, sunflower cake, hulled, defatted fish meal, dry, defatted bone meal, blood meal, egg powder,dry skimmed milk, hydrolized, irradiated yeast, and molasses), 10 milliliters of whole milk, 3 grams of carrots, and 1 gram of freshly clarified rendered lard, with a peroxide number of 0.006% iodine. The derived diet contained 20% albumin, 25% fat, 55% carbohydrates. The animals of the control group used pure fat without any additions; the animals of the 1st group were given pure fat with butyloxyanisole, and the animals of the 2nd group -- the same fat with butyloxytoluene. The antioxidant was added by calculating on 4 milligrams/

kilogram (aggravation of 10 times). In the control and in each of the experimental groups there were 10 animals in each.

Observation of the animals was carried out in accordance with the following tests: weight of the rats, cholesterol content and that of lipoid phosphorus in the blood serum, content of ketone bodies in the blood. To the extent that the part played by the liver in fat metabolism is very great, we made a rather detailed study of this organ. We made a determination of its weight, the ratio of the liver weight to the body weight, the content of dry substances, glycogen, fat, lipoid phosphorus in the liver, and the iodine number of the fat in the liver.

The cholesterol in the blood serum was determined by the colorimetric method, in accordance with the prescription of M. A. Levchenko (7). The lipoid phosphorus in the blood serum and in the liver was studied in accordance with Blyur (citation 8). In order to determine the ketone bodies, we made use of the distillation method, with a subsequent iodometric determination of the distilled-off acetone (9). The fat in the liver was determined in accordance with Sexhlet, by its extraction with petroleum ether, and the iodine number of the fat -- according to Gyubel (Hubel). The study of the glycogen in the liver was carried out by the Pfluger method in accordance with a prescription set forth in the paper of A.P. Belova (10).

As a result of the studies that had been carried out, it is possible to note the following.

The addition to fat of butyloxyanisole and of butyloxytoluene exerted no effect on the edibility of the fat and on the weight of the animals.

Acetone, beta-hydroxy butyric and acetoacetic acids represent normal products in the metabolism of higher fatty acids. In the norm, through the acetylized coenzyme A, and the cycle of tricarboxylic acids, they are rapidly oxidized to CO_2 and H_2O, and their content in the blood and in the urine is small.

((Two graphs on pg. 21 of text)) ((to the left ofthe graph at the left)): Cholesterol (in mg %); ((directly underneath this graph)): Days of the

experiment; ((below that)): Fig. 1 Cholesterol content in the blood serum in experimental and control animals (average data for the group). a -- animals that had received fat with butyloxyanisole; b -- animals that had received fat with butyloxytoluene; ((Russian)) v --control animals, which had received pure fat. ((within this graph, reading down)): ((Russian)) v; a; b. ((to the left of the graph at the right)): Lipoid phosphorus (in mg %); ((within this graph, reading down)): ((Russian)) b; a; v; ((directly underneath the same graph)): Days of the experiment; ((below that)): Fig. 2 Content of lipoid phosphorus in the blood serum in experimental and control animals (average data for the group).

The designations are the same as in Fig. 1

((text continued)): In reducing the oxidizing processes in the organism, possibly as a result of introducing, into the organism, rather active oxidation inhibitors, the content of ketone bodies in the blood could have grown. Hypoketonemia serves as an index of the insufficient oxidation of higher fatty acids in the liver, which was observed in affections of the liver. Over the extent of the entire experimental period, having investigated, three times, the content of ketone bodies in the blood of the rats, we did not disclose, in a single one of the periods, any increase or diminution of their amount in the blood (P is greater than 0.05). One could believe that the administration, into the organism of the animals, of antioxidants, in comparatively small doses, does not manifest any effect either on the formation, or on the oxidation of ketone bodies.

The level of cholesterol and lipoid phosphorus in the blood serum, in the animals of the experimental groups, over the entire extent of the experiment, remained the same as it was in the control animals. (Figs. 1 and 2). ((Graph on pg. 22 of text)) ((within this graph, reading from left to right)): a; b; ((Russian)) v; ((underneath this graph)): Fig. 3 Iodine number of the liver fat in the experimental and control animals. a -- animals that had received fat with butyloxyanisole; b -- animals that had

received fat with butyloxytoluene; v-- control animals, which had received pure fat. Dark circles -- iodine number of the liver fat in individual animals. The arithmetic mean value (M)for each group is designated by the columns with numberals. ((text continued))

In our previous investigations, over a period of feeding dogs butyloxyanisole and butyloxytoluene, we likewise did not disclose any increase in the cholesterol content in the blood (1). Thus, the experiments that we had conducted did not confirm any effect of the antioxidants under study on the cholesterol and lipoid phosphorus in the blood serum. Butyloxyanisole and butyloxytoluene were included in the diet in a dose aggravated by 10 times.

In the table are set forth indices that characterize the state of the liver in animals that had utilized fat along with butyloxyanisole and butyloxytoluene, and that in the controls.

Table

Results of Studying the Liver of Experimental and Control Animals

	Group of Animals				
	control	1st (received butyloxyanisole)		2nd (received butyloxytoluene)	
	$M_{control}$ plus or minus $m_{M_{control}}$	M_1 plus or minus m_{M_1}	P	P_2 plus or minus m_{M_2}	P
Weight of the liver (in grams)	9.18±0.32	9.04±0.44	>0.5	7.99±0.41	0.05
Ratio of the liver weight to the body weight (in %)	3.5±0.2	3.3±0.15	>0.25	3.2±0.1	0.1
Content of dry substances (in %)	29.9±0.3	29.7±0.3	>0.5	29.4±0.3	0.25
Glycogen(in gram %)	1.83±0.22	1.71±0.22	>0.5	1.72±0.18	>0.5
Lipoid phosphorus (in mg% for a dry substance)	216±7	225±9 >0.5	218±10	>0.25	
Fat (in gram % for a dry substance	17.8±0.6	17.3±0.6	0.5	18.0±0.3	>0.5

In animals that had received fat along with butyloxytoluene, there is a reduction in the weight of the liver (P = 0.05). However, in comparing the ratios of liver weight to the body weight (which, more correctly, characterizes the changes in the dimensions of the organ), it is evident that there is no statistically reliable difference between the experiment and the control. In the given case, one may merely speak of some tendency towards diminishing this ratio in the experimental animals.

The amount of dry substances, glycogen, lipoid phosphorus and fat, in the liver of the experimental animals, did not differ substantially from the controls.

The iodine number of fat in the liver (Fig. 3), which indicates the degree of its non-saturation, was reduced in the animals that had received fat along with butyloxytoluene (P = 0.05). This is in agreement with the data of Johnson and of his coauthors (4), who had also noted the reduction in the iodine number of fat in the liver, to be sure, while feeding butyloxytoluene in concentrations of 0.1 and 0.5%. In animals to whose diet was added butyloxyanisole, we did not disclose any statistically reliable difference (P is less than 0.1).

It is possible to believe that the reduction in the iodine number is associated with the fact that antioxidants inhibit the oxidation processes of fatty acids in the liver, possibly in the stage of dehydrogenation of the fatty acids, during which the saturated acetyl-coenzyme A converts to the $alpha_1$beta-unsaturated coenzyme A.

Conclusions

1. The feeding of butyloxyanisole over a period of 30 - 35 days exerted no influence on those aspects of fat metabolism in the organism that we had studied.

2. The feeding of fat along with butyloxytoluene brought about a reduction in the iodine number of fat in the liver in experimental animals; this attests to the fact that butyloxytoluene, apparently, manifests some

sort of influence on the oxidation of fatty acids in the organism.

3. With the purpose of selecting food inhibitors of oxidation that are the most effective and harmless for the organism, further studies are required with regard to the effect of possible toxic action of phenol antioxidants on the organism.

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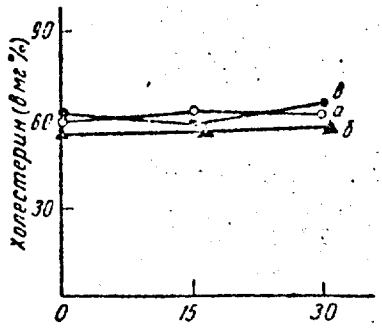

Fig. 1.

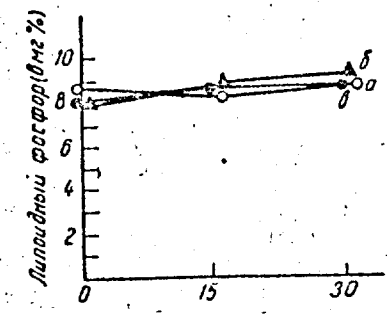

Fig. 2.

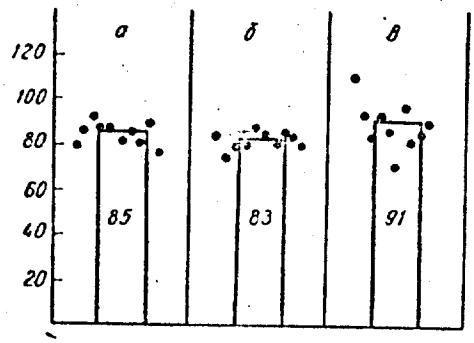

Fig. 3.

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Albino male-rats with an initial weight of 60—70 gm served as experimental animals. In two months the oil containing 4% of the oxidation products elicited in the animals a certain inhibition of growth, a statistically significant decline in the total amount of the blood serum protein, a reduced proportion of estherified liver cholesterol and a decreased activity of the alkaline phosphatase in the mucosa of the small intestine.

An analogous character of changes occurring in the values under study was also noted in the animals fed on sunflower oil subjected to a lesser degree of oxidation (containing 1.4% of oxidation products), but with a longer period of observation — up to 5 months.

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ВЛИЯНИЕ АНТИОКИСЛИТЕЛЕЙ ПИЩЕВЫХ ЖИРОВ (бутилоксианизола и бутилокситолуола) НА НЕКОТОРЫЕ СТОРОНЫ ЖИРОВОГО ОБМЕНА ЖИВОТНЫХ

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Защита жира и жиросодержащих продуктов от окисления, помимо большого экономического значения, является и гигиенической проблемой, поскольку продукты окисления и полимеризации жиров оказывают вредное действие на организм (токсическое, коканцерогенное, канцерогенное). Поэтому заслуживают внимания как физические, так и химические методы защиты жиров от окисления.

Поскольку окисление жира носит цепной характер, реакцию можно значительно замедлить с помощью ингибиторов окисления. Из синтетических антиокислителей наиболее эффективными и перспективными для применения в СССР являются бутилоксианизол, бутилокситолуол и эфиры галловой кислоты.

Вследствие того что антиокислители являются чуждыми организму химическими веществами, для использования в пищевой промышленности могут быть разрешены лишь абсолютно безвредные ингибиторы окисления, не изменяющие органолептических свойств продуктов.

На основании работ, проведенных в предыдущие годы на кафедре гигиены питания Центрального института усовершенствования врачей [1—3], бутилоксианизол и бутилокситолуол признаны сравнительно малотоксичными веществами. Небольшие их количества (0,01—0,02% по отношению к жиру рациона) в объеме проведенных исследований оказались безвредными для организма.

Однако в проблеме изучения возможного вредного действия синтетических антиокислителей на организм остается еще ряд интересных, но до сих пор недостаточно изученных вопросов, в том числе обмен в организме жира, содержащего антиокислители.

В литературе имеются указания, что антиокислители, по-видимому, влияют на процессы обмена жиров. В опытах Джонсона и соавторов [4], скармливавших крысам бутилоксианизол и бутилокситолуол, отмечено, что применение этих антиокислителей в количествах 0,1 и 0,5% по отношению к рациону повышает стабильность околопочечного жира крыс. Джонсон и Хьюджел [5] показали, что скармливание животным бутилоксианизола и особенно бутилокситолуола в концентрациях 0,3—0,5% приводит к увеличению содержания холестерина в сыворотке крови. Небольшие дозы бутилокситолуола (0,001%) не вызвали таких изменений. Дей и соавторы [6] сообщили о возможной взаимосвязи между метаболизмом бутилокситолуола (в относительно больших доз-

ровках), жирных кислот и транспортом холестерина и фосфолипидов. Упомянутые исследователи проводили опыты в основном с довольно большими дозами антиокислителей (агравация в 100—500 раз). Отечественных работ по влиянию фенольных антиокислителей на обмен жира нет.

Мы попытались проследить, как влияет скармливание жира с бутилоксианизолом и бутилокситолуолом на некоторые стороны жирового обмена.

Опыты ставили на крысах-самцах с первоначальным весом 120—150 г, которым давали в течение 30—35 дней следующий рацион: 15 г кормовых концентратов (содержащих муку пшеничную обойную, овсяную обойную, просяную, сухие пшеничные зародыши, подсолнечный жмых, очищенный от шелухи, обезжиренную рыбную муку, сухую костную обезжиренную муку, муку кровяную, яичный порошок, молоко снятое сухое, дрожжи гидролизные облученные, мелассу), 10 мл цельного молока, 3 г моркови и 1 г свиного топленого жира свежей вытопки, с перекисным числом 0,006% йода. Приведенный рацион содержал 20% белка, 25% жира, 55% углеводов. Животные контрольной группы потребляли чистый жир без добавок; животным 1-й группы давали чистый жир с бутилоксианизолом, животным 2-й группы — тот же жир с бутилокситолуолом. Антиокислитель добавляли из расчета 4 мг/кг (агравация в 10 раз). В контрольной и каждой из опытных групп было по 10 животных.

Обследование животных проводилось по следующим тестам: вес крыс, содержание холестерина и липоидного фосфора в сыворотке крови, содержание кетоновых тел в крови. Поскольку роль печени в обмене жира очень велика, мы довольно детально изучали этот орган. Определяли ее вес, отношение веса печени к весу тела, содержание сухих веществ, гликогена, жира, липоидного фосфора в печени, йодное число жира в печени.

Холестерин сыворотки крови определяли колориметрическим методом по прописи М. А. Левченко [7]. Липоидный фосфор в сыворотке крови и печени исследовали по Блюру [цит. 8]. Для определения кетоновых тел мы применили дистилляционный метод с последующим йодометрическим определением отогнанного ацетона [9]. Жир в печени определяли по Соклету, экстракцией его петролейным эфиром, йодное число жира — по Гюблю. Исследование гликогена в печени проводили методом Pflüger по прописи, приводимой в работе А. П. Беловой [10].

Полученные результаты обработаны методом вариационной статистики.

В результате проведенных исследований можно отметить следующее.

Добавление в жир бутилоксианизола и бутилокситолуола не влияло на поедаемость корма и вес животных.

Ацетон, β-оксимасляная и ацетоуксусная кислоты являются нормальными продуктами обмена высших жирных кислот. В норме они быстро через ацетилированный коэнзим А и цикл трикарбоновых кислот

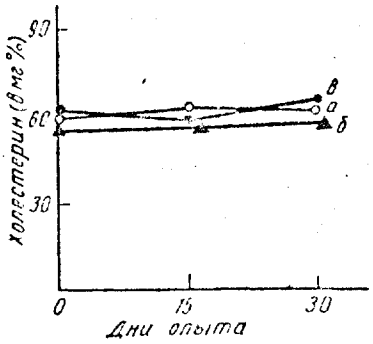

Рис. 1. Содержание холестерина в сыворотке крови у подопытных и контрольных животных (средние данные по группе).

а — животные, получавшие жир с бутилоксианизолом; *б* — животные, получавшие жир с бутилокситолуолом; *в* — контрольные животные, получавшие чистый жир.

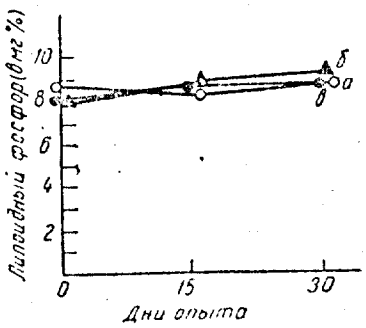

Рис. 2. Содержание липоидного фосфора в сыворотке крови у подопытных и контрольных животных (средние данные по группе).

Обозначения те же, что на рис. 1.

окисляются до CO_2 и H_2O и содержание их в крови и моче невелико. При снижении окислительных процессов в организме, возможном вследствие введения в организм довольно активных ингибиторов окисления, содержание кетоновых тел в крови могло возрасти. Гипокетонемия служит показателем недостаточного окисления высших жирных кислот в печени, наблюдающегося при поражениях печени. На протяжении опытного периода, трижды исследуя содержание кетоновых тел в крови крыс, мы ни в один из периодов не обнаружили увеличения или уменьшения их количества в крови ($P>0,05$). Можно думать, что введение в организм животных изучаемых антиокислителей в сравнительно небольших дозах не оказывает влияния ни на образование, ни на окисление кетоновых тел.

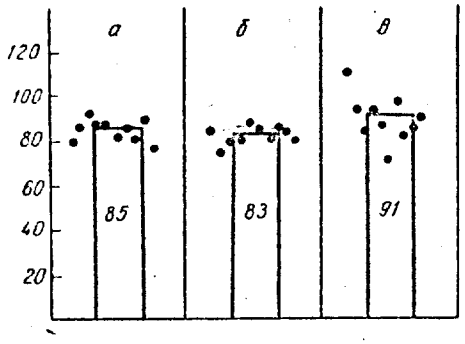

Рис. 3. Иодное число жира печени у подопытных и контрольных животных.

а — животные, получавшие жир с бутилоксианизолом; *б* — животные, получавшие жир с бутилокситолуолом; *в* — контрольные животные, получавшие чистый жир. Темные кружки — йодное число жира печени у отдельных животных. Столбиками с цифрами обозначена средняя арифметическая величина (*M*) по каждой группе.

Уровень холестерина и липоидного фосфора в сыворотке крови у животных опытных групп на всем протяжении опыта оставался таким же, как и у животных контрольных (рис. 1 и 2).

Мы в своих прежних исследованиях, в течение года скармливая собакам бутилоксианизол и бутилокситолуол, также не обнаружили увеличения содержания холестерина в крови [1]. Таким образом, проведенные нами опыты не подтвердили воздействия изучаемых антиокислителей на холестерин и липоидный фосфор в сыворотке крови. Бутилоксианизол и бутилокситолуол включали в рацион в дозе, агравированной в 10 раз.

В таблице представлены показатели, характеризующие состояние печени у животных, потреблявших жир с бутилоксианизолом и бутилокситолуолом, и у контрольных.

Результаты исследования печени подопытных и контрольных животных

Показатель	Группа животных				
	контрольная	1-я (получали бутилоксианизол)		2-я (получали бутилокситолуол)	
	$M_K \pm m_{M_K}$	$M_1 \pm m_{M_1}$	P	$P_2 \pm m_{M_2}$	P
Вес печени (в *г*)	9,18±0,32	9,04±0,44	>0,5	7,99±0,41	0,05
Отношение веса печени к весу тела (в %)	3,5±0,2	3,3±0,15	>0,25	3,2±0,1	0,1
Содержание сухих веществ (в %)	29,9±0,3	29,7±0,3	>0,5	29,4±0,3	0,25
Гликоген (в *г*%)	1,83±0,22	1,71±0,22	>0,5	1,72±0,18	>0,5
Липоидный фосфор (в *мг*% на сухое вещество)	216±7	225±9	>0,5	218±10	>0,25
Жир (в *г*% на сухое вещество)	17,8±0,6	17,3±0,6	0,5	18,0±0,3	>0,5

У животных, получавших жир с бутилокситолуолом, снижен вес печени ($P=0,05$). Однако при сравнении соотношений веса печени к весу тела (что более правильно характеризует изменения размеров органа) видно, что статистически достоверной разницы между опытом и контролем нет. В данном случае можно говорить только о некоторой тенденции к уменьшению этого соотношения у подопытных животных.

Содержание сухих веществ, гликогена, липоидного фосфора и жира в печени подопытных животных существенно не отличалось от контроля.

Йодное число жира в печени (рис. 3), которое показывает степень его ненасыщенности, было снижено у животных, получавших жир с бутилокситолуолом ($P=0{,}05$). Это согласуется с данными Джонсона и соавторов [4], также отмечавших снижение йодного числа жира в печени, правда, при скармливании бутилокситолуола в концентрациях 0,1 и 0,5%. У животных, в рацион которых добавляли бутилоксианизол, статистически достоверной разницы мы не выявили ($P>0{,}1$).

Можно думать, что снижение йодного числа связано с тем, что антиокислители ингибируют процессы окисления жирных кислот в печени, возможно, на этапе дегидрирования жирных кислот, при котором насыщенный ацетил-коэнзим А переходит в $\alpha_1\beta$-ненасыщенный коэнзим А.

Выводы

1. Скармливание в течение 30—35 дней бутилоксианизола не влияло на те стороны обмена жира в организме, которые мы изучали.

2. Скармливание жира с бутилокситолуолом привело к снижению йодного числа жира в печени у подопытных животных, что свидетельствует о том, что бутилокситолуол, по-видимому, оказывает какое-то влияние на окисление жирных кислот в организме.

3. С целью выбора наиболее эффективных и безвредных для организма пищевых ингибиторов окисления требуются дальнейшие исследования по выявлению возможного токсического действия фенольных антиокислителей на организм.

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THE EFFECT PRODUCED BY EDIBLE FATS ANTIOXIDANTS (BUTYLOXYANISOL AND BUTYLOXYTOLUOL) ON SOME ASPECTS OF FAT METABOLISM IN ANIMALS

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Summary

Subject to study was the effect produced by feeding fat together with butyloxyanisol and butyloxytoluol on some aspects of fat metabolism in rats. These antioxidants were given at a rate of 4 mg/kg body weigth over a period of 30—35 days. The animals were investigated for: their body weight, blood serum cholesterol and lipoid phosphorus, as well ketone body content, the weight of the liver, the ratio between the liver and total body weight, the content of dry substances, glycogen, fat, lipoid phosphorus and the iodine number of the liver fat.

Investigations have demonstrated that following butyloxyanisol feeding no changes occurred within the framework of the tests under study, whereas inclusion of butyloxytoluol in the diet of the animals resulted in the decline of the iodine number in the liver fat, which, as it appears, points to some influence exerted by butyloxytoluol on the oxidation of fatty acids in the organism.

Further research is needed to study the toxic action exercised on the organism by butyloxyanisol and butyloxytoluol.

concentration of α-methyltyramine accumulated per gram of heart was ten times higher than that present per ml in the perfusion medium, indicating that α-methyltyramine has a high affinity for the catecholamine uptake sites and is accumulated as rapidly as noradrenaline itself under similar conditions.

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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

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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% 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.

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.

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 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.

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 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.

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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Liver Response Tests. V. Effect of Dietary Restriction on a Short-term Feeding Study with Butylated Hydroxytoluene (BHT)*

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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.

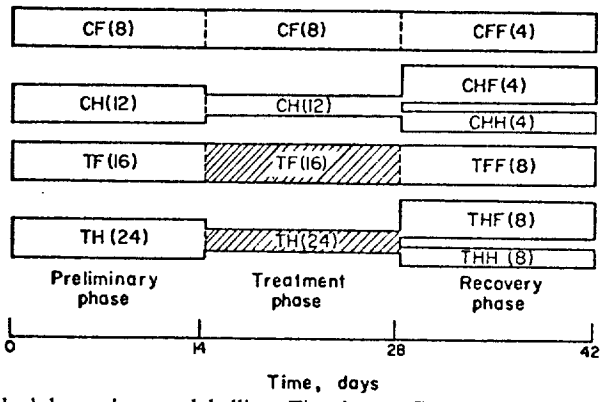

FIG. 1. Treatment schedules and group labelling. First letters C and T indicate control and BHT-treated respectively. Second letters F and H denote full and restricted diet during treatment phase. Third letters F and H signify full and restricted diet during recovery phase. Narrow bands indicate period 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).

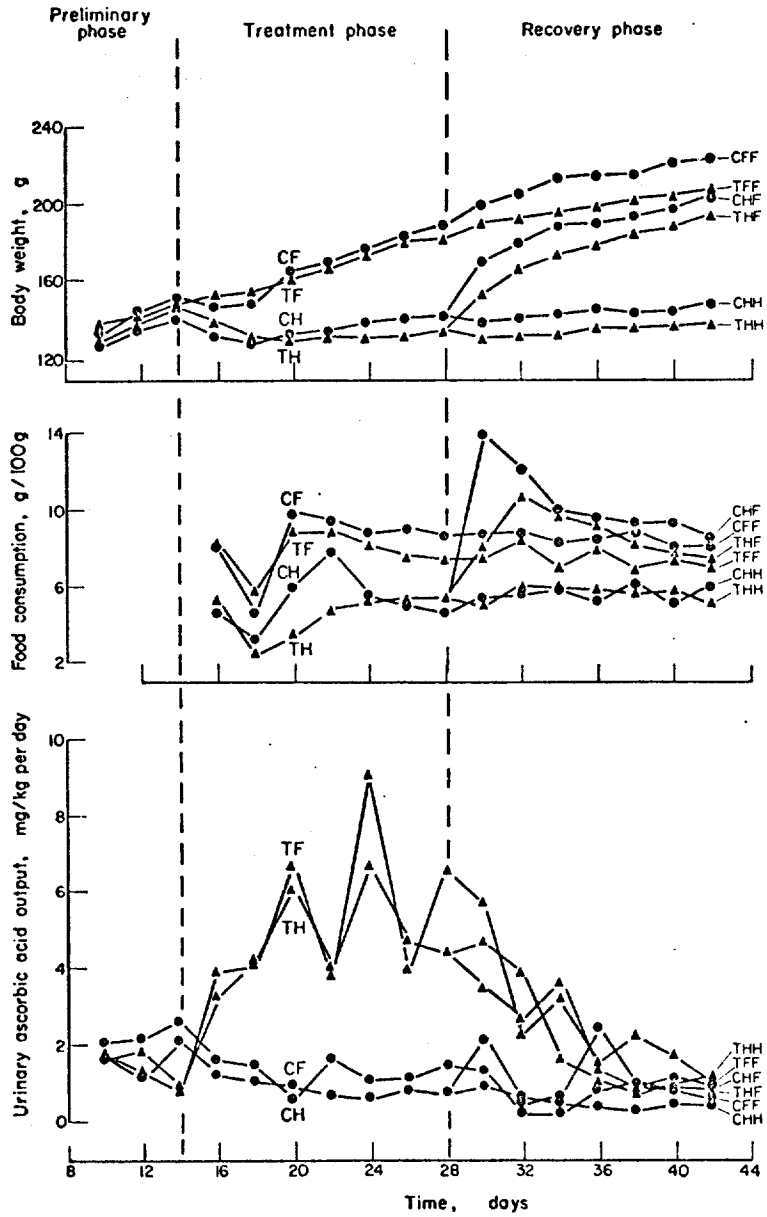

FIG. 2. Body weights, food consumption and daily output of urinary ascorbic acid of control rats (○) or rats treated with 500 mg BHT/kg (▲) and maintained on full or restricted diet, followed by a 14-day period of recovery as shown in Fig. 1. (Values of SE have been deliberately omitted to avoid confusion to the reader.)

Estimation of liver lipids. Samples of liver were homogenized in 0·73% (w/v) NaCl (Ansell & Hawthorne, 1964) and the homogenate diluted to 5 ml/g liver. Total cholesterol of the homogenate and esterified fatty acids and lipid phosphate of homogenate extracts were 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 only slight if any growth retardation (Fig. 2). The difference from control groups became more clearly manifest during the recovery phase, without reaching a level of significance ($P>0{\cdot}05$). Considering the fact that BHT-treated rats on restricted diet (group TH) consumed less food than the corresponding control group (CH) during the first week of treatment, and again on being restored to full diet (THF as compared with CHF), it must be concluded that BHT had no adverse effect on growth.

The effect of BHT on ascorbic acid excretion was striking, beginning almost at once and continuing at an enhanced level in all treated groups for about 1 wk after the cessation of 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 the administration of BHT (Fig. 3). On an absolute basis, liver weight was, of course, greatly 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

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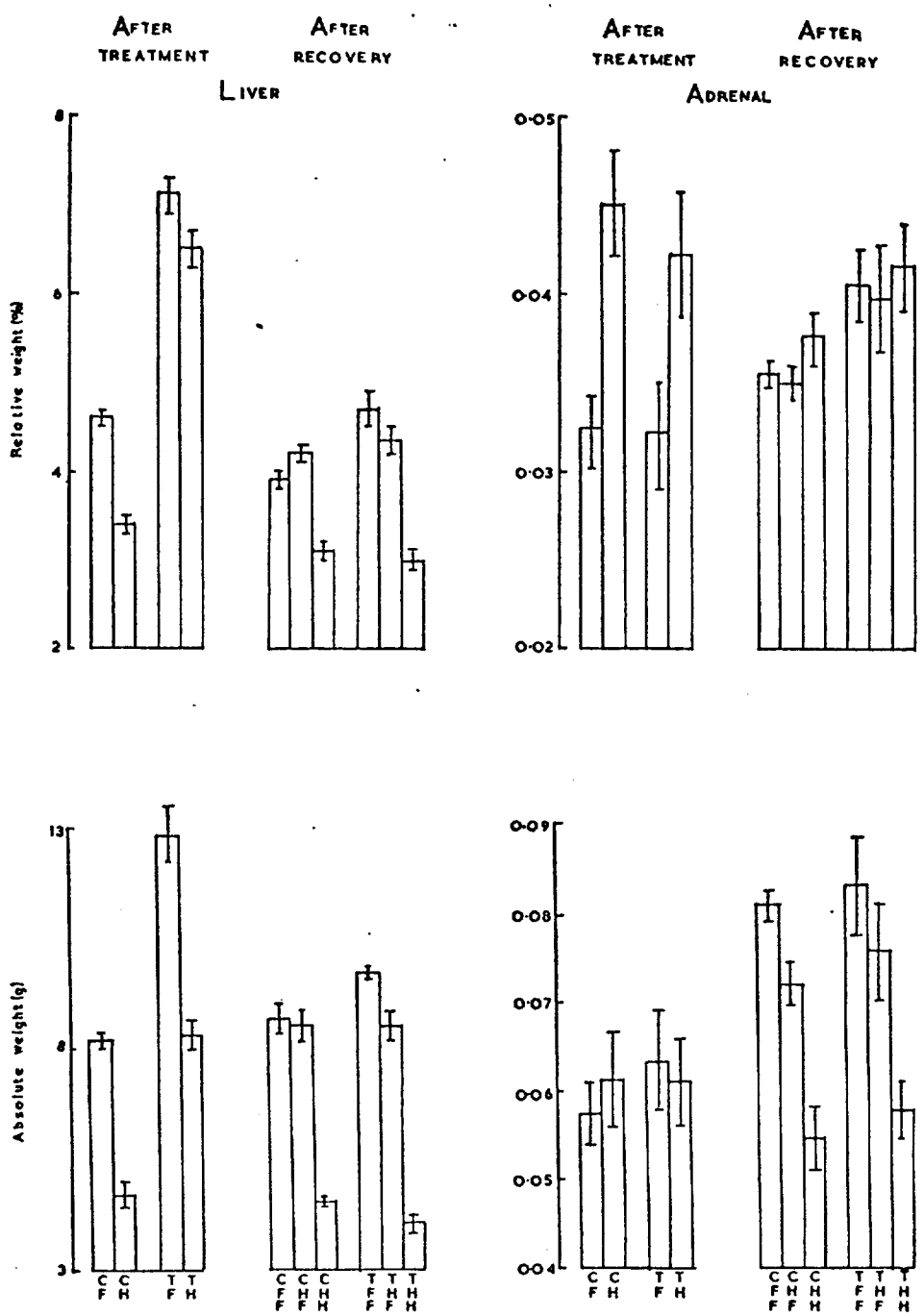

FIG. 3. Relative and absolute weights of liver and adrenals of control and BHT-treated rats receiving the various treatments as shown in Figs. 1 and 2. Vertical bars represent ±SE.

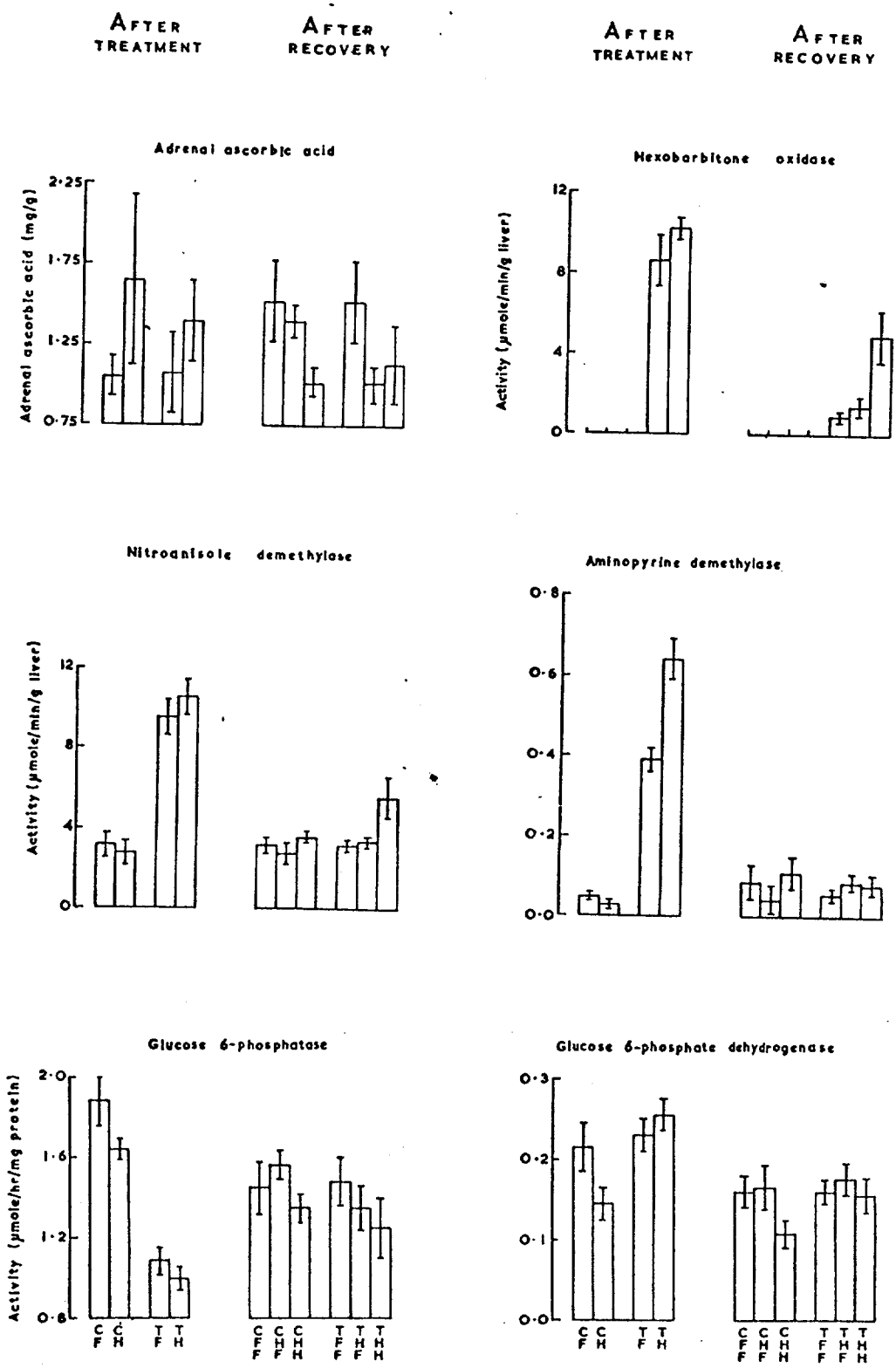

FIG. 4. Adrenal ascorbic acid content and activities of processing and other liver enzymes of control and BHT-treated rats receiving the various treatments shown in Figs. 1 and 2. The enzymes studied were hexobarbitone oxidase, nitroanisole demethylase and aminopyrine demethylase, glucose 6-phosphatase and glucose 6-phosphate dehydrogenase. Vertical bars represent ±SE.

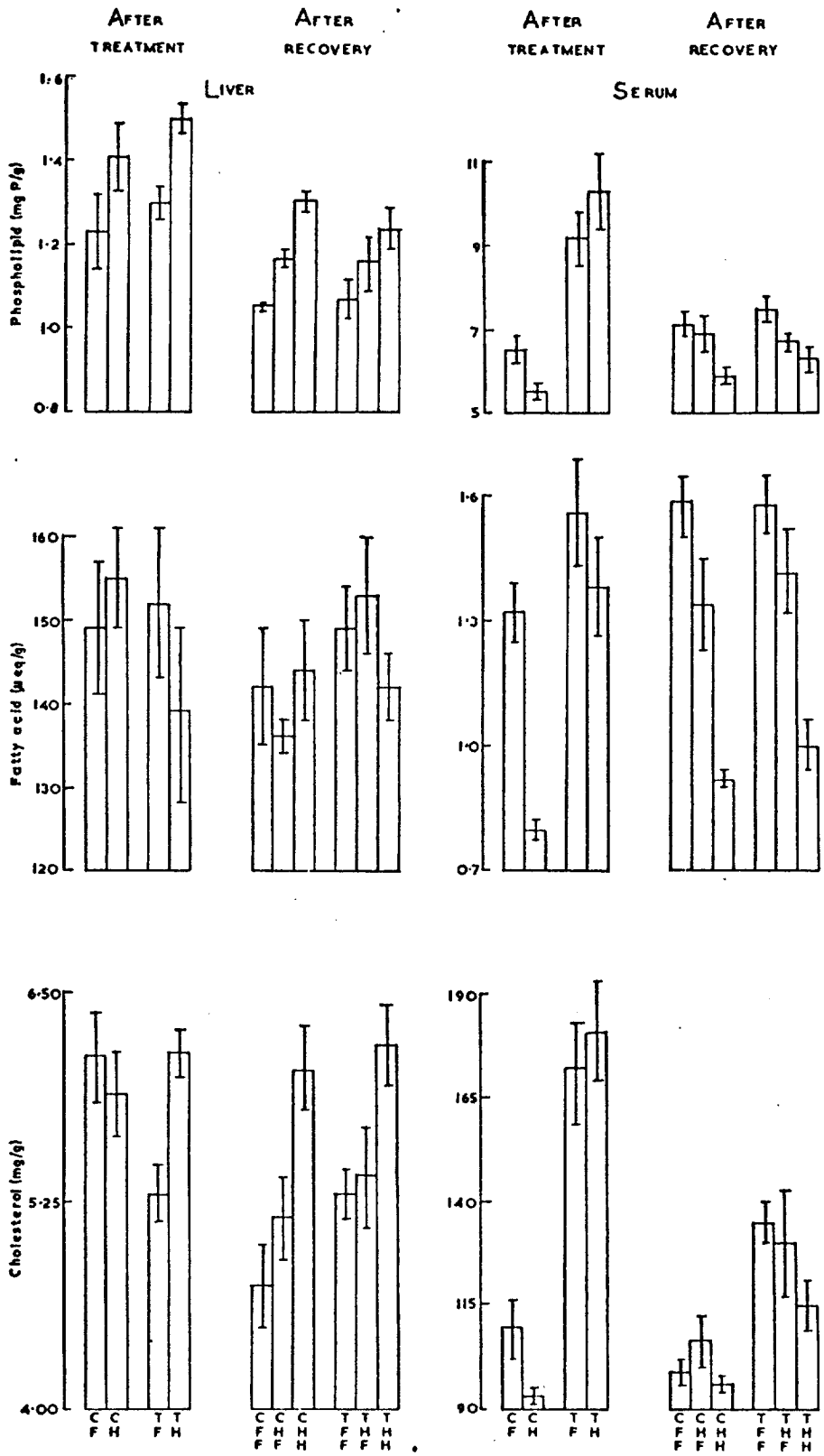

FIG. 5. Phospholipid, fatty acid and cholesterol levels of liver and serum in control and BHT-treated rats receiving the various treatments shown in Figs. 1 and 2. Vertical bars represent ±SE.

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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 18–24 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, Porcsalmy & 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 doses of BHT.

Effect of BHT on liver and serum lipid levels

A report of increased serum cholesterol and phospholipid levels was published by Day *et al.* (1959) who fed male rats on diets, with or without lard supplements, containing 0·5% BHT (dry weight basis) for 5 wk. In a longer study extending over more than 6 months, female rats given 0·1% BHT in a diet supplemented with 10% lard were found to have a serum cholesterol level 9% greater than that of controls on the same diet without BHT. That this increase (significant at the 5% level) was probably fortuitous is suggested by the absence of any corresponding increase in serum phospholipid and by the fact that a 20% lard supplement under the same conditions produced a fall of 8% (not significant) in serum cholesterol. Compare this result, for instance, with the shorter study on males using 0·5% BHT, in which both cholesterol and phospholipid levels were greatly enhanced by the 10% lard supplement and even more so by 20% lard. At the 0·1% level of BHT, the 5-wk study revealed a rise in serum cholesterol and phospholipid which was no longer evident with 10% or 20% lard. It is probably justifiable to conclude that in the experiments of Day *et al.* (1959) 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 rise of serum cholesterol and phospholipid was quite distinct in both fully-fed and diet restricted rats. After recovery, a slight elevation of serum cholesterol level persisted. It should be pointed out that these changes moved in parallel with the corresponding changes in relative liver weight, suggesting that the effects might be linked. From work carried out by Frawley, Kohn, Kay & Calandra (1965), it is clear that the incorporation of BHT in the diet of rats for 28 wk at levels at and below 1000 ppm has no effect on serum cholesterol. Only a slight increase was evident at the 3000 ppm level.

As well as reducing liver weight (Okey, Scheier & Reed, 1960; Mayfield & Roehm, 1961), overnight fasting of rats affects the liver lipids and elevates the serum cholesterol level and ratio of phospholipid to cholesterol (Mayfield & Roehm, 1961). The effect of fasting for 24 hr or more is to impair the capacity of the liver to incorporate acetate into cholesterol. The less severe conditions of food deprivation employed in the present study, while sufficient to reduce absolute and relative liver weight in control rats and to reduce serum fatty acids, had surprisingly little effect on liver lipids or other serum lipids.

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Tests de réaction hépatique. V. Effets de réductions alimentaires dans une etude de courte duree avec l'hydroxytoluène butylé (BHT)

Résumé—Une étude comportant des réductions alimentaires a été effectuée pour connaitre les effets de l'hydroxytoluène butylé (BHT; 3,5-di-*tert*-butyl-4-hydroxytoluène) sur la croissance, la consommation alimentaire, la teneur de l'urine et des surrénales en acide ascorbique, sur l'activité de plusieurs processus et enzymes hépatiques et sur les concentrations des lipides du foie et du sérum. Ces divers éléments ont fait l'objet de comparaisons entre des rats alimentés librement, et des groupes de rats recevant 50% de la ration de la veille des animaux témoins correspondants. Le BHT était administré chaque jour par tubage gastrique à raison de 500 mg/kg pendant 14 jours, cette période étant suivie par une période de repos de 14 jours.

Les rats traités au BHT avec et sans rationnement, se développaient aussi bien que les animaux témoins correspondants pendant toutes les périodes de traitement et de repos. On observait des augmentations parallèles du poids relatif du foie, des activités de l'aminopyrine déméthylase, de la nitroanisol déméthylase, et de l'hexobarbitone oxydase, ainsi que dans l'excretion urinaire d'acide ascorbique. La reduction de l'alimentation affectait extrêmement peu ces modifications et l'état initial se trouvait généralement complètement rétabli au bout de deux mois de repos. La diminution de l'activité de la glucose 6-phosphatase (sans modification de la glucose 6-phosphate déshydrogénase) constatée après le traitement au BHT était corrigée à la fin de la période de repos. Le poids et la teneur en acide ascorbique surrénales n'étaient pas modifiés par le traitement au BHT, même quand on superposait à ce traitement une réduction de l'alimentation.

Les proportions de phospholipides, d'acides gras libres et de cholestérol du foie n'étaient pas modifiées chez les rats qui avaient reçu du BHT avec ou sans réduction de l'alimentation. On constatait des augmentations frappantes des proportions de cholestérol et de phospholipides dans le sérum, dans les groupes traités au BHT. Ces augmentations disparaissaient après la période de repos.

Untersuchung der Leberreaktion. V. Einfluss der Futtereinschränkung auf eine kurzzeitige Verfütterung von butyliertem Hydroxytoluol (BHT)

Zusammenfassung—Der Einfluss der Verabreichung von butyliertem Hydroxytoluol (BHT; 3,5-Di-*tert*-butyl-4-hydroxytoluol) unter gleichzeitiger Futtereinschränkung auf Wachstum, Futterverbrauch, Ascorbinsäuregehalt im Urin und in der Nebenniere, Aktivität einer Anzahl arzneimittelmetabolisierender und anderer Enzyme der Leber und die Konzentration der Leber- und Serumlipide wurde untersucht. Es wurden die Ergebnisse von Ratten, die Futter *ad libitum* erhielten, verglichen mit Gruppen, denen 50% derjenigen Futtermenge angeboten wurde, die Kontrolltiere am vorangehenden Tage aufgenommen hatten. BHT wurde täglich mit einer Magensonde 14 Tage lang in einer Menge von 500 mg/kg verabreicht, worauf eine Erholungsperiode von 14 Tagen folgte.

Die mit BHT behandelten Ratten, welche die verminderte oder die volle Futtermenge erhielten, wuchsen gleichgut wie die entsprechenden Kontrolltiere, und zwar sowohl während der Behandlungs- als auch während der Erholungsperioden. Parallele Erhöhungen wurden beim relativen Lebergewicht, der Aktivität von Aminopyrindemethylase, Nitroanisoldemethylase und Hexobarbitaloxydase und der Ausscheidung von Ascorbinsäure im Urin beobachtet. Die Futtereinschränkung beeinflusste kaum diese Veränderungen, und im allgemeinen war der ursprüngliche Zustand nach 2 Wochen Erholung wiederhergestellt. Die Verminderung der Aktivität von Glucose-6-phosphatase (ohne Veränderung bei Glucose-6-phosphat-Dehydrogenase), die nach der BHT-Behandlung festgestellt wurde, war am Ende der Erholungsperiode in gleicher Weise wieder aufgehoben. Gewicht und Ascorbinsäuregehalt der Nebennieren blieben durch die BHT-Behandlung unbeeinflusst, selbst wenn Futtereinschränkung hinzukam.

Der Gehalt von Phospholipoid, freier Fettsäure und Cholesterin in der Leber blieb bei Ratten, die BHT mit oder ohne Futtereinschränkung erhielten, unbeeinflusst. Es gab auffallende Erhöhungen des Cholesterin- und Phospholipoidgehalts im Serum der mit BHT behandelten Gruppen; diese Erhöhungen waren nach der Erholung nicht mehr manifest.

(Barry, 1964)), followed by infection with the virus led to virus replication and an increase in the rate of RNA synthesis. Sendai and Melbourne viruses were then inactivated by ultraviolet irradiation, and Semliki Forest and Chikungunya virus replication prevented by incubating the cells at 42° after infection at 37° (Ruiz-Gomez & Isaacs, 1963). These viruses produced interferon, but not virus, in cells untreated with actinomycin. In actinomycin treated cells they caused no increase in the rate of RNA synthesis as compared with controls, and it is concluded that viral nucleic acid synthesis is not necessary for interferon production.

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The Association of [6,7-$^3H_2$]Oestradiol with Nuclear Chromatin

By R. J. B. King, J. Gordon and L. Martin. (*Imperial Cancer Research Fund, Lincoln's Inn Fields, London, W.C. 2*)

Current theories suggest that the primary action(s) of oestrogens is within the nucleus (Mueller, 1965). It might therefore be expected that in 'target tissues', oestrogen would be located primarily in the nucleus. The intracellular distribution of [6,7-$^3H_2$]oestradiol has been studied in oestrogen-responsive tissues. Anterior pituitary, uterus and dimethylbenzanthracene-induced mammary tumour were obtained from rats 1hr. after injection of 0·1μg. of [6,7-$^3H_2$]oestradiol/100g. body wt. Mouse vaginae were isolated 30min. after local application of $5\times10^{-5}\mu$g. of [6,7-$^3H_2$]oestradiol. With the exception of uterine epithelium, over 60% of the tissue radioactivity can be isolated with the nuclei from these tissues. In uterine epithelium, 30–40% was associated with the nuclei.

Extraction of tumour nuclei with 0·1M-tris, pH 7·4, removed negligible amounts of [6,7-$^3H_2$]-oestradiol whilst extraction with 2M-NaCl removed 67% of the steroid. Chromatography of this extract on Sephadex G-100 indicated that the [6,7-^3H]$_2$-oestradiol was extracted in association with macromolecules. This suggested that the steroid might be associated with the deoxyribonucleoprotein complex. Extraction of tumour nuclei with buffers between pH 8 and 3 showed that the binding site was acid labile. Steroid was extracted in increasing amounts when the pH fell below 7. At pH 3, 90% of the [6,7-$^3H_2$]oestradiol was removed. No change in extraction of nuclear proteins was noted over this pH range. Equilibrium dialysis experiments suggested that the receptor molecule was not a histone and may be acidic protein.

After sonic disruption of tumour nuclei, 60% of the nuclear radioactivity was associated with the chromatin fraction. A similar result was obtained with nuclei from anterior pituitary. Equal amounts of [6,7-$^3H_2$]oestradiol were present in the chromatin and 10^5g soluble fractions of nuclei from uterine epithelium. Nuclei from vaginae were more resistant to sonic disruption but the results so far obtained are compatible with the idea that oestradiol is associated with chromatin. No evidence has been found that the [6,7-$^3H_2$]oestradiol is associated with any particular type of chromatin.

With the possible exception of uterine epithelium these results are compatible with the view that oestradiol affects cellular function by associating with an acid-labile, non-histone component of the nuclear chromatin.

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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±

11 p.p.m. respectively. Despite continued administration of BHT, over the next two days the level in 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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Additional Evidence of the Identity of Rat Liver Microsomal Glucose 6-Phosphatase and Inorganic Pyrophosphatase

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It has been found by Nordlie & Arion (1964) that glucose 6-phosphatase and inorganic pyrophosphatase activities of rat liver microsomes could not be separated by fractionation. These enzymes showed similarities with respect to some kinetic measurements and the effects of various treatments (Stetten & Taft, 1964; Fisher & Stetten, 1965). These results do not provide conclusive evidence of the involvement of a single enzyme. Since both are microsomal enzymes, it is possible that glucose 6-phosphatase and pyrophosphatase are equally susceptible to various treatments. In view of the observations that administration of various hepatotoxic compounds to rats decreased the activity of liver glucose 6-phosphatase (Feuer, Golberg & Le Pelley, 1964; Feuer, Golberg & Le Pelley, 1965) it was of interest to discover whether the same is true of inorganic phosphatase activity.

Both enzymes were significantly reduced in the livers of rats treated with coumarin (200mg./kg. in arachis oil daily for seven days by gastric intubation); the loss of activity was proportional to the dose of coumarin. In liver microsomes isolated from coumarin-treated rats the two enzymes were also affected equally, compared with microsomes from rats given arachis oil.

Previous experiments demonstrated that *o*-tyrosine acts as a selective activator of liver glucose 6-phosphatase (Feuer, Golberg & Gibson, 1964*a*) and *o*-hydroxyphenylacetic acid is a potent inhibitor (Feuer, Golberg & Gibson, 1964*b*). Inorganic pyrophosphatase has been found to be affected in a like manner by *o*-tyrosine and by *o*-hydroxyphenylacetic acid. Prolonged dialysis of microsomes against sucrose–EDTA–phosphate solution results in a loss in the activity of both enzymes. This loss could be prevented by the addition of *o*-tyrosine to the dialysis medium.

It appears from these investigations that *o*-tyrosine is necessary for the function of both enzymes. Nevertheless, the presence of this compound in mammalian liver does not appear to have been demonstrated previously. Starting from rat liver microsomes we have isolated *o*-tyrosine by column and thin-layer chromatography (Gaitonde, 1961) and identified it as the diazo compound coupled with *p*-nitroaniline and as the dinitrophenyl derivative.

The involvement of a phosphoenzyme intermediate has been suggested for rat-liver glucose 6-phosphatase (Segal, 1959; Hass & Bryne, 1960) and evidence has been provided for the formation of such an intermediate by yeast inorganic pyrophosphatase (Cohn, 1958). It may be postulated that *o*-tyrosine functions as a phosphate acceptor and that formation of *o*-tyrosine-*O*-phosphate is involved in the action of the liver microsomal enzyme.

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Liver Response Tests. III. Liver Enlargement and Stimulation of Microsomal Processing Enzyme Activity*

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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: * $P<0·05$; ** $P<0·01$; *** $P<0·001$.

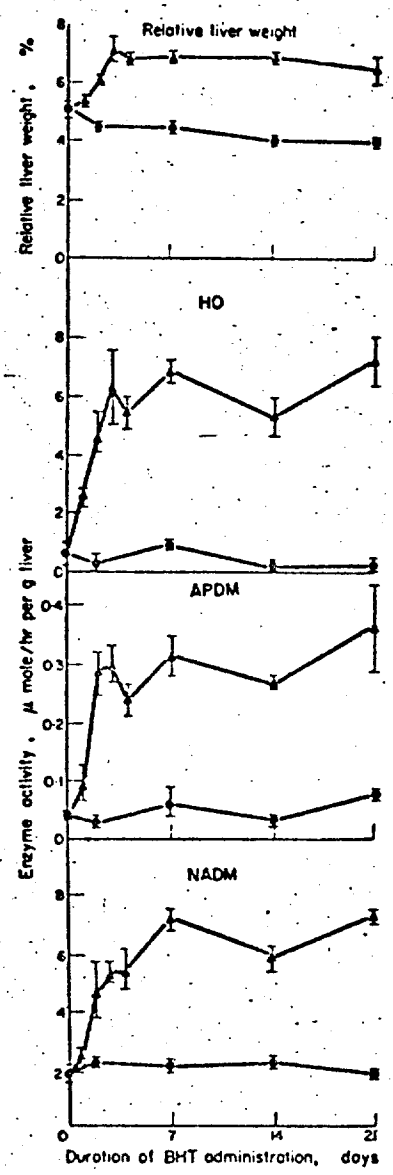

FIG. 1a. Time course of the effect of BHT on relative weight and processing enzyme activities of female rats. Carworth rats were given BHT (▲) at 500 mg/kg or arachis oil (●) daily by stomach tube. Vertical bars indicate SE. Each group comprised 4 rats except at 2 days (6 rats) and 3 days (3 rats).

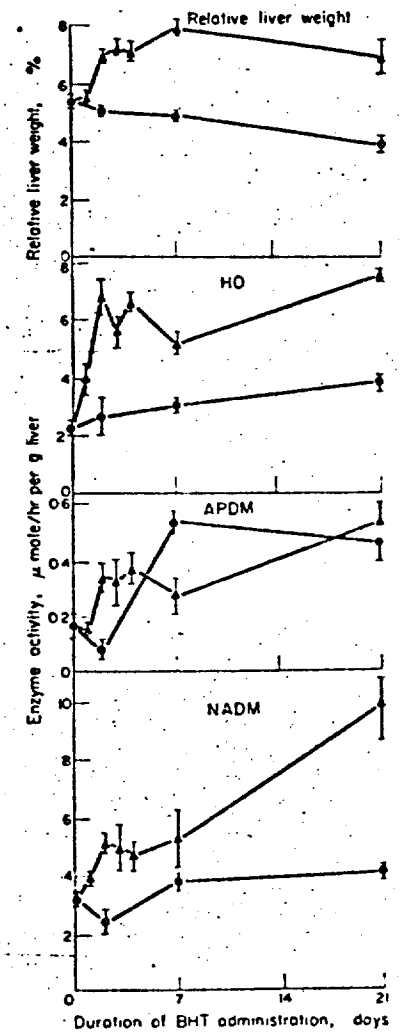

FIG. 1b. Time course of the effect of BHT on relative liver weight and processing enzyme activities of male rats. Carworth rats were given BHT (▲) at 500 mg/kg or arachis oil daily by stomach tube. Vertical bars indicate SE. Each group comprised 4 rats except at 2 days (6 rats), 7 days (6 rats) and 21 days (2 rats).

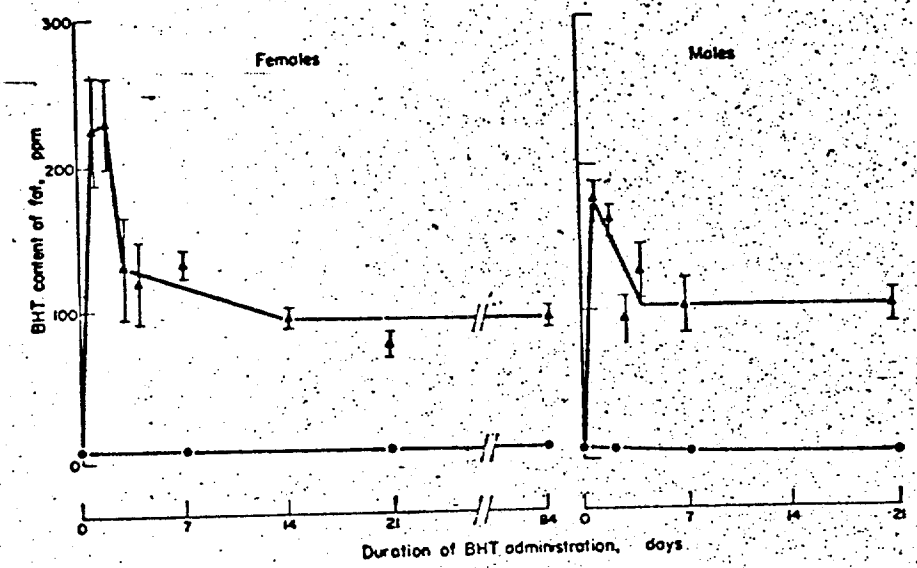

FIG. 2. Changes with time of the BHT content of abdominal fat. For details see legends to Figs. 1a and 1b.

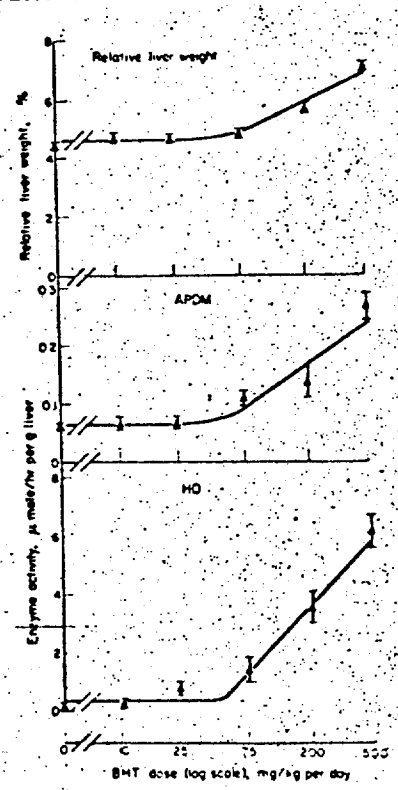

FIG. 3. Effect of various doses of BHT on relative liver weight and processing enzyme activities. Female Carworth rats were given BHT (10–500 mg kg daily) for 7 days by stomach tube. Vertical bars indicate SE. Each group comprised 4–6 rats. BHT dose is plotted on a logarithmic scale.

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).

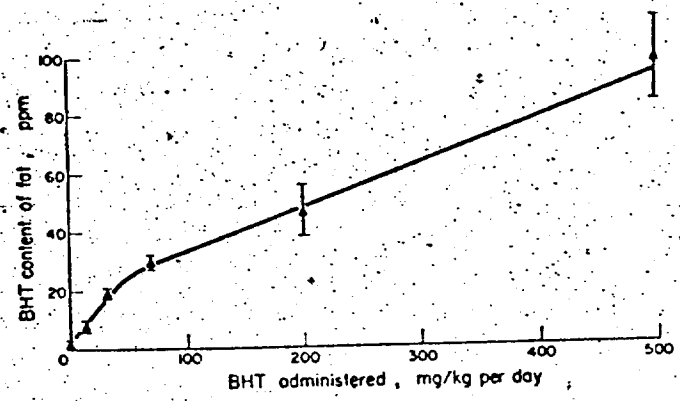

FIG. 4. Effect of various doses of BHT on the BHT content of abdominal fat. BHT dose is plotted on a linear scale. For other details see legend to Fig. 3.

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'etude 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 Mausen festgestellt. Mit butyliertem Hydroxyanisol (BHA; 3-*tert*-butyl-4-hydroxyanisol) behandelte Ratten liessen zwar eine Lebervergrösserung, aber keine Veränderung der Aktivitat 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-

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Liver Response Tests. I. Exploratory Studies on Glucose 6-phosphatase and Other Liver Enzymes

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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 β-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 6×10^3; mitochondria, $3{\cdot}3 \times 10^4$; lysosomes, $2{\cdot}5 \times 10^5$; microsomes, 3×10^6.

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±0·94
			Female	0·198±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±0·64
			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	3·64±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 text. 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	100–200
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 ±0·13
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*

	Duration of treatment (days)			
	9		16	
Enzyme	Control	Barbitone	Control	Barbitone
Acid phosphatase	0·116±0·010	0·129±0·011	0·114±0·008	0·146±0·009
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	Enzyme: 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 6–18 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±0·03***	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
Drazine [(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
Sucroglyceride	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±0·045	6·26±0·22***
Sodium barbitone	200 mg	Intraperitoneal	9–16 days	2·15±0·14	0·243±0·030	5·50±0·24

Rats: 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: *$P<0·05$; **$P<0·02$; ***$P<0·01$.

2-Phenylindole	250 mg	Oral	7–14 days	1·14±0·15***	0·459±0·045	6·26±0·22***
Sodium barbitone	200 mg	Intraperitoneal	2–16 days	2·15±0·14	0·243±0·030	5·50±0·24

Rats: 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±0·060	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	0·480±0·126	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***	0·88±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	1·93±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***	0·444±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. β-Glucuronidase and cathepsin were unchanged during intoxication with carbon tetrachloride, coumarin or safrole, but when allyl alcohol was injected β-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

cytoplasmic 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 provoquèrent 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'entraîna 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 festzustellen.

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-dimethylaminoazobenzol, Dimethylnitrosamin, Aflatoxin), mit Nahrungsmittelzusätzen (Butyliertes Hydroxyanisol, Butyliertes Hydroxytoluol, Sucroglyceridl), mit Stabilisatoren (Dioctylzinn, 2-Phenylindol), mit Medikamenten (Drazin, Natriumäthylbarbiturat) 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.

A Study of the Enzyme Secretory Function of the Small Intestine and Pancreas Mucosa in Rats Receiving Phenol Antioxidants in Their Diet

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The prdection of alimentary fats and fat-containing products from oxidation by means of introducing antioxidants is receiving constantly greater circulation both in our country as well as abroad. Some of the proposed antioxidants have been studied from the toxilogical aspect and have been authorized for use in the food industry.

All the same, many aspects of the organism's activity, upon introducing antioxidants with food have not yet been studied. The problem as to what influence is had by food containing antioxidants on the enzymatic activity of the gastrointestinal tract has not yet been clarified sufficiently. It is known that the enzyme scretory function of the digestive glands is closely associated with the character of nutrition (1 - 4); in varying the chemical composition of food, the glands are capable of finely (delicately) regulating the amount and composition of the secretions being produced(5).

The purpose of the present investigation has been to study the effect of a number of antioxidants of the phenolic series on the activity of the enterokinase and of the alkaline phosphatase in the mucosa (mucous membrane) of the small intestine, where their concentration is the greatest, and also on the secretion of the pancreatic enzymes (lipase and amylase).

A study was made of 5 antioxidants (butyloxyanisol, butyloxytoluol, propyl- and dodecylgallates) and of a mixture of antioxidants (butyloxyanisol with propylgallate).

For the experiment there were taken 160 white male mice with an initial weight of 80 - 90 grams. The experiments were carried out in 4 series. In the I and II

series the antioxidants were fed over a period of 1 month, and in the III and IV series -- 3 months. In the experiment, altogether, there were 12 experimental and 4 control groups. The experimental (test) animals received, daily, 15 grams of a No. 1 nutritive feed concentrate(cracked wheat flour, and also, cracked oatmeal and millet flour, dry wheat seeds, hulled sunflower oil cakes, defatted fish flour, defatted dry bone meal, blood meal, egg powder, skimmed dry milk, hydrolyzed irradiated yeast, molasses), 10 milliliters of whole (unskimmed) milk, 3 grams of carrots and 1 gram of rendered lard, freshly melted, with a peroxide number of 0.006% iodine. The enumerated diet, with respect to caloricity, comprised 20% albumen, 25% fat, and 55% carbohydrates.

In the I and II series of experiments, the animals of the 1st group were given, in their diet, along with the rendered lard, butyloxyanisol (BOA); those of the 2nd group -- butyloxytoluol (BOT); those of the 3rd group -- dodecylgallate (DG); the animals of the 4th group represented the control (K). In the II and IV series of experiments,the animals of the 1st group received in their diet ethylgallate (EG; those of the 2nd group -- propylgallate (PG); those of the 3rd group -- a mixture of butyloxyanisol and propylgallate (BOA plus PG); the 4th group represented the control (K). The corresponding antioxidants were included in the diet of a fixed group, calculating 4 milligrams for 1 kilogram of body weight of the animal (a tenfold aggravation). A mixture of BOA and PG was fed in the same dose, but in a ratio of 2 : 1 (the most effective ratio for protecting alimentary fats and products from oxidation). The animals of the control group received the same diet, but the rendered lard, which was included in the feed, was free from antioxidants. The difference between I and III, II and IV series consisted merely in the duration of feeding. The duration of the experiments in the I and II series was 1 month, and in the III and IV series it was 3 months.

The activity of the enterokinase and alkaline phosphatase was determined in a scraping from the mucosa of the small intestine. For this purpose, following the experiment, the animals were destroyed by decapitation; from the upper section

(superior portion) of the small intestine a section was removed that had a length of 10 centimeters. After washing off the intestine from its contents by means of the polished (ground) edge of a (microscope) slide, a scraping was made from the mucous membrane (mucosa). Having weighed it, it was placed in a glass homogenizer in which there was prepared a water extraction diluted to 1 : 20. The extraction continued for 2 hours at room temperature. From the centrifugate thus obtained (centrifugal separation of 10 - 15 minutes at 3000 revolutions per minute) there was prepared a dilution of 1 : 1000, which was employed for determining the enterokinase and alkaline phosphatase. The enterokinase content was determined by the method proposed by G. K. Shlygin and it was expressed in arbitrary units for 1 gram of weight.

The activity of the alkaline phosphatase was determined in accordance with the method recommended by L. S. Fomina, and it was likewise expressed in arbitrary units for 1 gram of weight. The enzymatic activity of the pancreas was studied by means of determining the amylase content in the blood and the lipase (content) in the pancreas tissue. The amylase was investigated by the method of Engel'gardt (Engelhardt) and Gerchuk in the animals, while still alive, prior to the beginning of the experiment, and in the 15, 30, 45 - 50 and 85 - 95th day of the experimental period, and its activity was expressed in milligrams for 1 milliliter.

The lipase content was determined in the tissue of the pancreas immediately after decapitation and in the dissection of the abdominal cavity of the animals. Following extraction, the gland was weighed and pulverized in a mortar with distilled water (dilution of 1 : 20) with the addition of finely ground glass. The extraction continued for 30 minutes at room temperature. Following a 5-minute centrifuge separation, at the rate of 1500 revolutions per minute, there was prepared a dilution of 1 : 20000, which served as an initial one for determining the lipase in accordance with the method of G. K. Shlygin, L. S. Fomina and Z. M. Pavlova. The lipase activity was expressed in arbitrary units for 1 gram of weight. The results thus obtained were treated by the method of statistics of variations.

During the period of the experiments, the animals appeared healthy, neat

(clean), and consumed the entire feed. The increase in weight in the experimental and control groups was practically identical.

In Tables 1 and 2 are set forth the results of determining the content of the enterokinase in the mucous membrane (mucosa) of the small intestine while introducing, into the feed of the animals, over a course of 1 and 3 months, fat with antioxidants.

((Two tables on pg. 22 of text))

Table 1

Enterokinase Activity in a Scraping from the Mucous Membrane (Mucosa) of the Superior Portion of the Small Intestine in Rats That Received, Over the Course of 1 Month, in Their Diet, Fat with Antioxidants

Series of Experiments	Group of Animals	Antioxidant	M	sigma	m_M	t	P
I	1st	Butyloxyanisol	((See Table 1 on pg. 22 of text for all figures pertaining thereto))				
	2nd	Butyloxytoluol					
	3rd	Dodecylgallate					
	4th	Control					
II	1st	Ethylgallate	((See Table 1 for figures, etc.))				
	2nd	Propylgallate					
	3rd	Butyloxyanisol plus					
	4th	Control					

Table 2

Activity of Enterokinase in a Scraping of Mucosa from the Superior Portion of the Small Intestine in Rats Receiving in Their Diet, Over a Period of 3 Months, Fat with Antioxidants (in units/gram)

Series of Experiments	Group of Animals	Antioxidant	M	sigma	m_M	t	p
III	1st	Butyloxyanisol	((See Table 2 on pg. 22 of text for all figures pertaining thereto)				
	2nd	Butyloxytoluol					
	3rd	Dodecylgallate					
	4th	Control					

((Table continued on the next page))

((Table continued from preceding page)

IV	1st	Ethylgallate	
	2nd	Propylgallate	
	2a-	Control with PG	((See Table 2 for figures, etc.))
	3rd	Butyloxyanisol plus propylgallate	
	4th	Control	

((text continued)): The enterokinase activity in the control groups comprised 2600 plus or minus 300 units/gram (I series) and 3400 plus or minus 340 units/gram (II series). In animals that had received antioxidants, the enterokinase activity was approximately within the same range: in the I series, 2600 - 2750 units/gram, and in the II series, 3500 - 3900 units/gram. There was somewhat of an increase, in our opinion, in the enterokinase activity in the group of animals that had received PG (M = 4000 units/gram), but in this group there is a rather high array (series) dispersion (sigma = plus or minus 1300) and the difference, on the whole, with respect to the group, is unreliable (P is greater than 0.25).

((Graph on pg. 23 of text)) ((in the upper left-hand corner of this graph)): units/gram; ((within this graph)): BOT (butyloxytoluol); K (control); ((underneath this graph)): Fig. 1. Enterokinase content in the scraping from the mucosa of the superior portion of the small intestine in control rats and in those that had received, over a period of 3 months, fat with butyloxytoluol. BOT -- animals that had received fat with butyloxytoluol; K -- control animals that had received pure fat. Dark circles -- enterokinase activity in individual animals. Here, and in Figs. 2, 3: columns with numbers -- arithmetical mean (M) for each group.

((text continued)):

A three-month introduction, in the feed, of a majority of the antioxidants under study, also showed no noticeable effect on the enterokinase content. Statistically, a reliable decrease in the amountof this enzyme has been noted in the group of animals that had received BOT (the enterokinase activity in the control group was 3100 plus or minus 200 units/gram; in the experimental group, 2500 plus or minus 130 units/gram). In spite of the fact that in statistical treatment reliable results had been obtained, one may assume that there is no great difference in the activity of the enzyme (Fig. 1).

In determining the activity of the alkaline phosphatase in the scraping from the mucosa of the small intestine, with a single-month feeding of antioxidants, no distinctions were disclosed between the experimental and control groups of animals (Table 3). ((Table on pg. 23 of text))

Table 3

Activity of the Alkaline Phosphatase in a Scraping from the Mucosa of the Superior Portion of the Small Intestine in Rats that had Received, over the Period of 1 Month, in Their Diet, Fat with Antioxidants (in units/gram)

Series of Experiments	Group of Animals	Antioxidant	M	sigma	m_M	t	P
I	1st	Butyloxyanisol	((See Table 3 on pg. 23 of text for all figures pertaining thereto))				
	2nd	Butyloxytoluol					
	3rd	Dodecylgallate					
	4th	Control					
II	1st	Ethylgallate	((See Table 3 for figures))				
	2nd	Propylgallate					
	3rd	Butyloxyanisol plus propylgallate					
	4th	Control					

((text continued)): A three-month administration of antioxidants into the organism of animals had no noticeable reflection on the activity of the alkaline phosphatase in the mucosa of the superior portion of the small intestine (Table 4). It is only in the group of animals that had received EG (ethylgallate) that we noted a statistically reliable reduction in the activity of the alkaline phosphatase.

((Table on pg. 24 of text))

Table 4

Activity of the Alkaline Phosphatase in a Scraping from the Mucosa of the Superior Portion of the Small Intestine in Rats that had Received, in the Course of 3 Months, in Their Diet, Fat with Antioxidants (in units/gram)

((Table continued on next page))

((table continued from the preceding page))

Series of Experiments	Group of Animals	Antioxidant	M	sigma	m_M	t	P
III	1st	Butyloxyanisol	((See Table 4 on pg. 24 of text for all figures pertaining thereto))				
	2nd	Butyloxytoluol					
	3rd	Dodecylgallate					
	4th	Control					
IV	1st	Ethylgallate	((See Table 3 for figures))				
	2nd	Propylgallate					
	2a -	Control with PG					
	3rd	Butyloxyanisol plus propylgallate					
	4th	Control					

((text continued)): In this manner, a month's administration of antioxidants into the organism of animals showed no influence on the enterokinase activity nor on that of the alkaline phosphatase.

With a 3-month administration of antioxidants only in the group of animals that had received EG (ethylgallate), there was noted a reduction in the activity of this enzyme. The difference disclosed in the enterokinase activity in the BOT group can hardly be taken into consideration in view of the fact that, by proceeding from the concept concerning the limit of physiological fluctuations in the activity of this enzyme, we cannot consider this difference as being an essential one.

((Graph on pg. 24 of text)) ((in the upper part of this graph, reading from left to right and then down and again from left to right)): units/gram; I Series, II Series; BOA (butyloxyanisol); BOT (butyloxytoluol); DG (dodecylgallate; K (Control); EG (ethylgallate); PG (propylgallate); BO A plus PG; K (Control; ((alongside of this graph, at lower right)): Fig. 2. Lipase activity in the tissue of the pancreas in rats in whose diet, over the course of 1 month, there was included fat with antioxidants.

((text continued)): Investigations carried out on the blood amylase have shown that the activity of this enzyme, which has been determined over the course of a number of days in succession, in various animals, and even in the same animals,

shows a very marked variation. In the control animals, which had not been subjected to a possible harmful effect of antioxidants, the amylase activity was from 60 to 180 milligrams/milliliter. The same rat from the control group, over an extension of several days, yielded the following indices for the amylase activity of the blood: 62 - 90 - 180 - 83 milligrams/milliliter.

In all the experimental groups, the fluctuation limit in individual animals was also very high. In connection with this, we believe that this index, which was not constant in its quantitative characteristic, represents no particular interest for us.

((Graph on pg. 25 of text)) ((within this graph, at the top, reading across and then down and across again)): units/gram; I Series; II Series; BOA; BOT; DG; K (control); EG; PG; BOA plus PG; K (control); ((to the left of this graph, at the lower left)): Fig. 3. Lipase activity in the tissue of the pancreas in rats that had received, over the course of 3 months, in their diet,fat with antioxidants. ((text continued)):

In Figs. 2 and 3 are shown the results of determining the lipase activity in the tissue of the pancreas while feeding antioxidants over a period of 1 and 3 months. The lipase content in the individual groups is given in the form of averages (means). An analysis of this material attests to the fact that there is no significant difference in the experimental groups in comparison with the control (one); this is confirmed also by the results of the statistical treatment of the material. The existing difference in some groups should be considered as immaterial (in all cases, P is greater than 0.25).

F. E. Budagyan and L. I. Smirnova (6) have shown that the inclusion of antioxidants in the diet of the animals brings about some reduction in the assimilability of nutritive substances, especially of fats (the difference was not very substantial -- 0.5 - 0.9%). The authors, in their paper, did not explain the mechanism of the possible diminution in the assimilability. In setting up the present study, we wished to track down whether there was not a reduction in the absorption of the nutritive substances by the intestinal wall as a result of a disturbance in the secretory function of the digestive glands. As has been shown

by the investigations carried out, the majority of the antioxidants that we have studied (BOA, PG, DG (dodecylgallate), BOA plus PG), administered per os in the course of 1 and 3 months, do not indicate any influence on the enterokinase content nor on that of the alkaline phosphatase in the mucosa of the small intestine.

Some reduction in the activity of the alkaline phosphatase, which was noted in the study, in animals that had received EG (ethylgallate), and of the enterokinase in animals to whom BOT (butyloxytoluol) had been administered, is possible associated with the fact that the inclusion, in the food, of these antioxidants, while changing the usual chemical composition of the food, brings about, by reflex, a compensatory change in the enzyme secretory function of the intestine.

The lipase content in the tissue of the pancreas did not vary in a single one of the 12 experimental groups of animals. These investigations made it possible to reject the assumption with respect to the reduction in the assimilability of the fat that contained antioxidants, as a result of diminishing the secretion of the lipolytic enzyme in the pancreas.

A determination of the amylase activity in the blood of rats indicated a very high variability in this index in all groups, including those under control. We assume that the index, which yields high physiological fluctuations, cannot be employed as a sensitive test for studying the possible toxic action of various chemical additions on the organism.

Conclusions

1. The introduction into the diet, in the course of 1 and 3 months, of some antioxidants of the phenol series has indicated no effect on the enterokinase activity in the mucosa of the superior portion of the small intestine in white rats.

2. The content of the alkaline phosphatase in the mucosa of the small intestine showed no variation in animals that had received antioxidants with their feed over a period of 1 month. The feeding of ethylgallate, over a period of 3 months, led to some diminution in the activity of the alkaline phosphatase in the mucos of the small intestine. The remaining 5 antioxidants under investiga-

3. The activity of the pancreatic enzymes (lipase and amylase) showed no significant variation in the case of feeding antioxidants over a period of 1 and 3 months.

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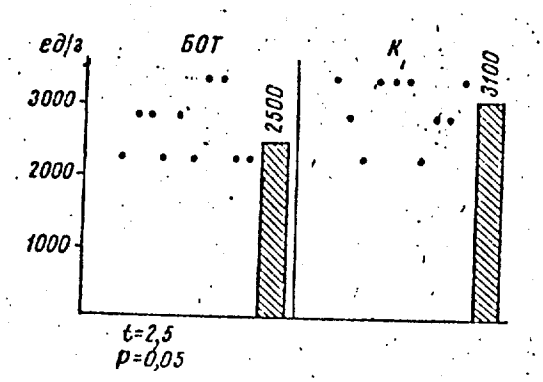

Figure 1

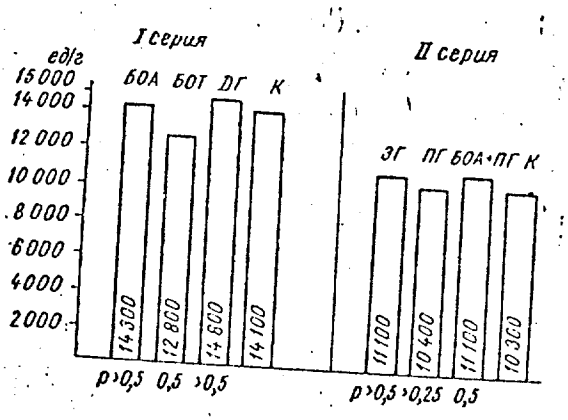

Figure 2

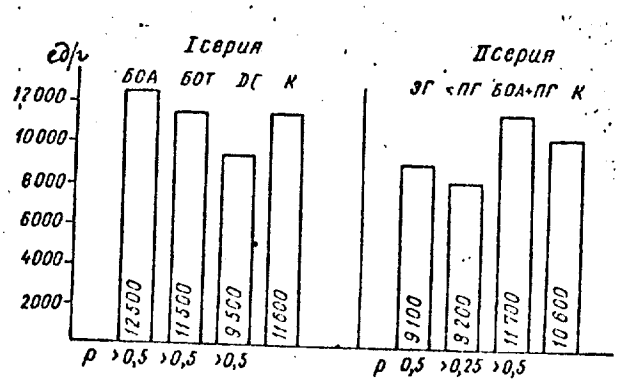

Figure 3

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ИЗУЧЕНИЕ ФЕРМЕНТОВЫДЕЛИТЕЛЬНОЙ ФУНКЦИИ СЛИЗИСТОЙ ОБОЛОЧКИ ТОНКОГО КИШЕЧНИКА И ПОДЖЕЛУДОЧНОЙ ЖЕЛЕЗЫ У КРЫС, ПОЛУЧАВШИХ В РАЦИОНЕ ФЕНОЛЬНЫЕ АНТИОКИСЛИТЕЛИ

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Защита пищевых жиров и жирсодержащих продуктов от окисления путем внесения антиоксидантов находит все большее распространение и у нас, и за рубежом. Некоторые из предлагаемых антиокислителей изучены в токсикологическом аспекте и разрешены для использования в пищевой промышленности.

Вместе с тем многие стороны деятельности организма при введении антиокислителей с пищей еще не изучены. Вопрос о том, как влияет пища, содержащая антиокислители, на ферментативную деятельность желудочно-кишечного тракта, освещен недостаточно. Известно, что ферментовыделительная функция пищеварительных желез тесно связана с характером питания [1—4]; при изменении химического состава пищи железы способны тонко регулировать количество и состав вырабатываемых секретов [5].

Целью данного исследования явилось изучение влияния ряда антиокислителей фенольного ряда на активность энтерокиназы и щелочной фосфатазы в слизистой оболочке тонкой кишки, где их концентрация наиболее высока, а также на секрецию панкреатических ферментов (липазы и амилазы).

Было изучено 5 антиокислителей (бутилоксианизол, бутилокситолуол, этил-, пропил-, додецилгаллаты) и смесь антиокислителей (бутилоксианизол с пропилгаллатом).

В опыт было взято 160 белых крыс-самцов с начальным весом 80—90 г. Опыты проведены в 4 сериях. В I и II серии антиокислители скармливались в течение 1 месяца, в III и IV серии — 3 месяца. Всего в опыте было 12 опытных и 4 контрольные группы. Подопытные животные получали ежедневно 15 г пищевого кормового концентрата № 1 (пшеничная обойная мука, а также овсяная обойная и просяная мука, сухие пшеничные зародыши, очищенный от шелухи подсолнечный жмых, обезжиренная рыбная мука, обезжиренная сухая костная мука, кровяная мука, яичный порошок, снятое сухое молоко, гидролизные облученные дрожжи, меласса), 10 мл цельного молока, 3 г моркови и 1 г свиного топленого жира свежей вытопки с перекисным числом 0,006% йода. Приведенный рацион по калорийности составлял 20% белка, 25% жира, 55% углеводов.

В I и III серии опытов животным 1-й группы давали в рационе со свиным топленым жиром бутилоксианизол (БОА), 2-й группы — бутилокситолуол (БОТ), 3-й — додецилгаллат (ДГ); животные 4-й группы являлись контрольными (К). Во II и IV серии опытов животные 1-й группы получали в рационе этилгаллат (ЭГ), 2-й — пропилгаллат (ПГ), 3-й — смесь бутилоксианизола и пропилгаллата (БОА+ПГ); 4-я группа являлась контрольной (К). Соответствующие антиокислители включали в рацион определенной группы из расчета 4 мг на 1 кг веса тела животного (аггравация в 10 раз). Смесь БОА и ПГ скармливали в этой же дозе, но в соотношении 2 : 1 (наиболее эффективное соотношение для защиты пищевых жиров и продуктов от окисления). Животные контрольных групп получали тот же рацион, но включаемый в корм свиной топленый жир был свободен от антиокислителей. Разница между I и III, II и IV сериями заключалась только в продолжительности скармливания. Продолжительность опытов I и II серии 1 месяц, III и IV серии 3 месяца.

Активность энтерокиназы и щелочной фосфатазы определяли в соскобе со слизистой оболочки тонкого кишечника. Для этого после опыта животных забивали декапитацией, из верхнего отдела тонкого кишечника отделяли отрезок длиной 10 см. После отмывания кишечника от содержимого отшлифованной гранью предметного стекла делали соскоб со слизистой оболочки. Взвесив, его помещали в стеклянный гомогенизатор, где готовили водную вытяжку в разведении 1 : 20. Экстракция продолжалась 2 часа при комнатной температуре. Из полученного центрифугата (центрифугирование 10—15 мин. при 3000 об/мин) готовили разведение 1 : 1000, которое использовали для определения энтерокиназы и щелочной фосфатазы. Содержание энтерокиназы определяли по методу, предложенному Г. К. Шлыгиным, и выражали в условных единицах на 1 г веса.

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Активность щелочной фосфатазы определяли по методу, рекомендованному Л. С. Фоминой, и выражали также в условных единицах на 1 г веса. Ферментативную деятельность поджелудочной железы изучали путем определения содержания амилазы в крови и липазы в ткани поджелудочной железы. Амилазу исследовали методом Энгельгардта и Герчука у животных прижизненно до начала эксперимента, на 15, 30, 45—50 и 85—95-й день опытного периода и активность ее выражали в миллиграммах на 1 мл.

Содержание липазы определяли в ткани поджелудочной железы сразу же после декапитации и вскрытия брюшной полости животных. После извлечения железу взвешивали и растирали в ступке с дистиллированной водой (разведение 1:20) с добавлением мелко измельченного стекла. Экстракция продолжалась 30 мин. при комнатной температуре. После 5-минутного центрифугирования со скоростью 1500 об/мин готовили разведение 1:20 000, которое служило исходным для определения липазы по методу Г. К. Шлыгина, Л. С. Фоминой и З. М. Павловой. Активность липазы выражали в условных единицах на 1 г веса. Полученные результаты обрабатывали методом вариационной статистики.

В период опытов животные выглядели здоровыми, опрятными, съедали весь корм. Прибавка веса в опытных и контрольных группах осуществлялась практически одинаково.

В табл. 1 и 2 приведены результаты определения содержания энтерокиназы в слизистой оболочке тонкого кишечника при введении

Таблица 1

Активность энтерокиназы в соскобе со слизистой оболочки верхнего отдела тонкого кишечника у крыс, получавших в течение 1 месяца в рационе жир с антиокислителями (в *ед/г*)

Серия опытов	Группа животных	Антиокислитель	M	σ	m_M	t	P
I	1-я	Бутилоксианизол	2 750	700	300	0,38	>0,5
	2-я	Бутилокситолуол	2 600	600	250	0	1
	3-я	Додецилгаллат	2 600	600	250	0	1
	4-я	Контроль	2 600	600	250		
II	1-я	Этилгаллат	3 900	870	360	1,0	>0,25
	2-я	Пропилгаллат	4 000	1300	530	1,0	>0,25
	3-я	Бутилоксианизол + пропилгаллат	3 500	650	270	0,2	>0,5
	4-я	Контроль	3 400	880	360		

Таблица 2

Активность энтерокиназы в соскобе слизистой оболочки верхнего отдела тонкого кишечника у крыс, получавших в течение 3 месяцев в рационе жир с антиокислителями (в *ед/г*)

Серия опытов	Группа животных	Антиокислитель	M	σ	m_M	t	P
III	1-я	Бутилоксианизол	3 100	480	200	0	1
	2-я	Бутилокситолуол	2 500	320	130	2,5	0,05
	3-я	Додецилгаллат	2 700	640	260	1,2	0,25
	4-я	Контроль	3 100	480	200		
IV	1-я	Этилгаллат	3 100	800	330	0,2	>0,5
	2-я	Пропилгаллат	3 800	270	110	0,3	>0,5
	2а-я	Контроль ПГ	3 700	620	250		
	3-я	Бутилоксианизол + пропилгаллат	3 600	990	400	1,0	>0,25
	4-я	Контроль	3 160	360	150		

в течение 1 и 3 месяцев животным корма, содержащего жир с антиокислителями.

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Активность энтерокиназы в контрольных группах составляла 2600±300 ед/г (I серия) и 3400±360 ед/г (II серия). У животных, получавших антиокислители, активность энтерокиназы была приблизительно в тех же пределах: в I серии 2600—2750 ед/г, во II серии 3500—3900 ед/г. Несколько увеличена, по нашему мнению, активность энтерокиназы в группе животных, получавших ПГ (M=4000 ед/г), но в этой группе довольно велика разбросанность ряда ($\sigma=\pm1300$) и разница целиком по группе недостоверна ($P>0{,}25$).

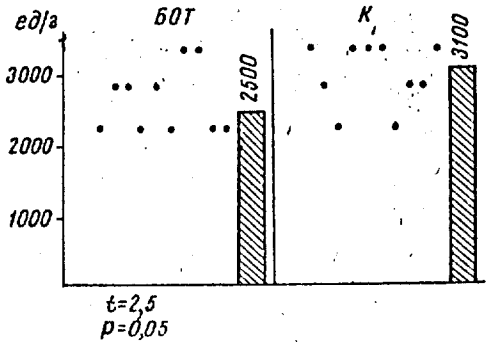

Рис. 1. Содержание энтерокиназы в соскобе со слизистой оболочки верхнего отдела тонкого кишечника у контрольных крыс и получавших в течение 3 месяцев жир с бутилокситолуолом.

БОТ — животные, получавшие жир с бутилокситолуолом; *К* — контрольные животные, получавшие чистый жир. Темные кружки — активность энтерокиназы у отдельных животных. Здесь и на рис. 2, 3: столбики с цифрами — средняя арифметическая (M) по каждой группе.

Трехмесячное введение в корм большинства изучаемых антиокислителей также не оказывало заметного влияния на содержание энтерокиназы. Статистически достоверное уменьшение количества этого фермента отмечено в группе животных, получавших БОТ (активность энтерокиназы в контрольной группе 3100±200 ед/г; в опытной группе 2500±130 ед/г). Несмотря на то что при статистической обработке получены достоверные результаты, можно считать, что большой разницы в активности фермента нет (рис. 1).

При определении активности щелочной фосфатазы в соскобе со слизистой оболочки тонкого кишечника при одномесячном скармливании антиокислителей не было обнаружено различий между опытными и контрольными группами животных (табл. 3).

Трехмесячное поступление антиокислителей в организм животных заметно не отразилось на активности щелочной фосфатазы в слизистой

Таблица 3

Активность щелочной фосфатазы в соскобе слизистой оболочки верхнего отдела тонкого кишечника у крыс, получавших в течение 1 месяца в рационе жир с антиокислителями (в *ед/г*)

Серия опытов	Группа животных	Антиокислитель	M	σ	m_M	t	P
I	1-я	Бутилоксианизол	33 800	15 500	6 300	1,7	>0,1
	2-я	Бутилокситолуол	39 700	11 100	4 500	1,1	>0,25
	3-я	Додецилгаллат	40 500	8 500	3 500	1,1	>0,25
	4-я	Контроль	46 800	10 650	4 350		
II	1-я	Этилгаллат	43 900	13 600	5 800	0,8	>0,25
	2-я	Пропилгаллат	41 100	15 000	6 100	1,2	0,25
	3-я	Бутилоксианизол + пропилгаллат	42 700	15 000	6 100	0,9	>0,25
	4-я	Контроль	48 400	3 640	1 500		

оболочке верхнего отдела тонкого кишечника (табл. 4). Только в группе животных, получавших ЭГ, мы отметили статистически достоверное понижение активности щелочной фосфатазы.

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Таблица 4

Активность щелочной фосфатазы в соскобе слизистой оболочки верхнего отдела тонкого кишечника у крыс, получавших в течение 3 месяцев в рационе жир с антиокислителями (в *ед/г*)

Серия опытов	Группа животных	Антиокислитель	*M*	σ	m_M	*t*	*P*
III	1-я	Бутилоксианизол	57 800	17 800	7 300	0,9	>0,25
	2-я	Бутилокситолуол	54 000	16 600	6 800	1,2	0,25
	3-я	Додецилгаллат	54 900	26 600	10 800	1	>0,25
	4-я	Контроль	70 800	30 800	12 200		
IV	1-я	Этилгаллат	24 700	10 400	4 250	2,2	0,05
	2-я	Пропилгаллат	46 000	8 100	3 300	0,12	>0,5
	2а-я	Контроль ПГ	46 900	16 800	6 850		
	3-я	Бутилоксианизол + пропилгаллат	45 200	18 000	7 350	0,2	>0,5
	4-я	Контроль	47 500	22 750	9 300		

Таким образом, месячное введение антиокислителей в организм животных не оказывало влияния на активность энтерокиназы и щелочной фосфатазы.

При 3-месячном введении антиокислителей только в группе животных, получавших ЭГ, было отмечено снижение активности этого фермента. Выявленная разница в активности энтерокиназы в группе БОТ вряд ли может быть принята во внимание, так как, исходя из представ-

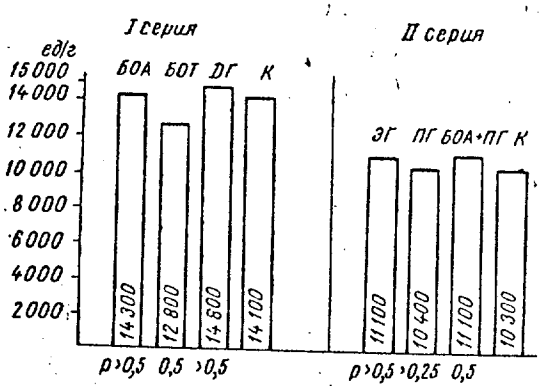

Рис. 2. Активность липазы ткани поджелудочной железы у крыс, в рацион которых в течение 1 месяца включали жир с антиокислителями.

ления о пределе физиологических колебаний активности этого фермента, мы не можем рассматривать эту разницу как существенную.

Проведенные исследования амилазы крови показали, что активность этого фермента, определенная в течение ряда дней подряд, у различных животных и даже у одних и тех же животных очень сильно варьирует. У контрольных животных, не подвергающихся возможному вредному влиянию антиокислителей, активность амилазы была от 60 до 180 мг/мл. Одна и та же крыса контрольной группы на протяжении нескольких дней давала следующие показатели амилазной активности крови: 62—90—180—83 мг/мл.

Во всех опытных группах предел колебаний у отдельных животных был также очень велик. В связи с этим мы считаем, что этот непостоянный в своей количественной характеристике показатель не представляет для нас особого интереса.

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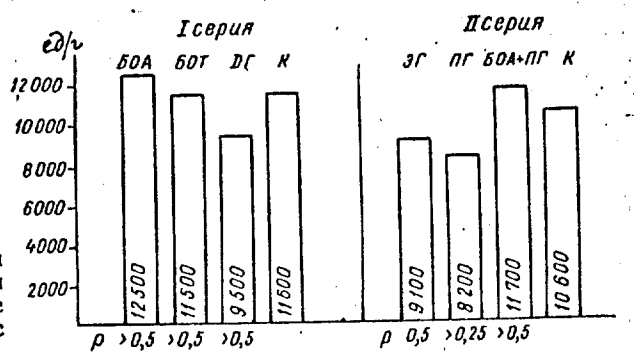

Рис. 3. Активность липазы ткани поджелудочной железы у крыс, получавших в течение 3 месяцев в рационе жир с антиокислителями.

На рис. 2 и 3 показаны результаты определения активности липазы в ткани поджелудочной железы при скармливании антиокислителей в течение 1 и 3 месяцев. Содержание липазы в отдельных группах дано в виде средних. Анализ этого материала говорит о том, что значительной разницы в опытных группах по сравнению с контролем нет, что подтверждается также результатами статистической обработки материала. Имеющаяся разница в некоторых группах должна рассматриваться как несущественная (во всех случаях $P>0{,}25$).

Ф. Е. Будагян и Л. И. Смирнова [6] показали, что включение в рацион животных антиокислителей ведет к некоторому уменьшению усвояемости пищевых веществ, особенно жиров (разница была не очень существенна — 0,5—0,9%). Авторы в своей работе не объяснили механизма возможного уменьшения усвояемости. При постановке данного исследования нам хотелось проследить, не является ли уменьшение всасывания пищевых веществ кишечной стенкой результатом нарушения секреторной функции пищеварительных желез. Как показали проведенные исследования, большинство изучаемых нами антиокислителей (БОА, ПГ, ДГ, БОА + ПГ), вводимых per os в течение 1 и 3 месяцев, не оказывает влияния на содержание энтерокиназы и щелочной фосфатазы в слизистой оболочке тонкого кишечника.

Отмечаемое в работе некоторое уменьшение активности щелочной фосфатазы у животных, получавших ЭГ, и энтерокиназы у животных, которым вводили БОТ, возможно, связано с тем, что включение в пищу этих антиокислителей, изменяя привычный химический состав пищи, рефлекторно приводит к компенсаторному изменению ферментовыделительной функции кишечника.

Содержание липазы в ткани поджелудочной железы не изменилось ни в одном из 12 опытных групп животных. Эти исследования позволили отвергнуть предположение о снижении усвояемости жира, содержащего антиокислители, за счет уменьшения секреции липолитического фермента поджелудочной железой.

Определение активности амилазы в крови у крыс показало очень большую вариабельность этого показателя во всех группах, в том числе и в контрольных. Мы полагаем, что показатель, дающий большие физиологические колебания, не может быть использован как чувствительный тест для изучения возможного токсического действия различных химических добавок на организм.

Выводы

1. Введение в рацион в течение 1 и 3 месяцев некоторых антиокислителей фенольного ряда не оказывало влияния на активность энтерокиназы в слизистой оболочке верхнего отдела тонкого кишечника белых крыс.

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2. Содержание щелочной фосфатазы в слизистой оболочке тонкого кишечника не было изменено у животных, получавших в течение 1 месяца с кормом антиокислители. Скармливание этилгаллата в течение 3 месяцев привело к некоторому уменьшению активности щелочной фосфатазы в слизистой оболочке тонкого кишечника. Остальные 5 изучаемых антиокислителей такого действия не оказывали.

3. Активность панкреатических ферментов (липазы и амилазы) существенно не изменялась при скармливании антиокислителей в течение 1 и 3 месяцев.

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Поступила 19/VI 1967 г.

A STUDY OF THE ENZYME SECRETORY FUNCTION OF THE SMALL INTESTINE AND PANCREAS MUCOSA IN RATS FED ON A DIET CONTAINING PHENOL ANTIOXIDENTS

I. A. Karplyuk (Moscow)

Summary

Study was made of the action exerted by a series of phenol antioxidents (butyloxyanisol, butyloxytoluene, ethyl-, propyl-, dodethylgallates and a mixture of butyloxyanisol and propylgallate) introduced per os to rats during one and three months, on the activity of enterokinase and alkaline phosphatase in the small intestine mucosa, and also on the secretion of pancreatic enzymes (amylase and lipase).

The effected research did not show any changes in the development of enterokinase in the mucosa of the superior portion of the small intestine. A diminished production of phosphatase by the small intestine wall was noted in the group of animals receiving ethylgallate for 3 months. One and three months long feeding of antioxidents does not affect the activity of the lipolytic enzyme secreted by the pancreas.

УДК 612.333:[612.397+612.397.23

ЭКСКРЕЦИЯ НЕЙТРАЛЬНЫХ ЖИРОВ И СВОБОДНЫХ ЖИРНЫХ КИСЛОТ СТЕНКОЙ ТОНКОГО КИШЕЧНИКА В ПРОЦЕССЕ ПИЩЕВАРЕНИЯ У СОБАК

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Выделение различных веществ в полость пищеварительного тракта имеет большое физиологическое значение [1—5]. Значительный интерес представляет выделение различных липидов в пищеварительном тракте. Так, детально изучено выведение фосфолипидов с желчью [6—8], с кишечным соком [4—5, 9—12]. Полагают, что выведение фосфолипидов в полость пищеварительного тракта в какой-то степени обеспечивает печень липотропными факторами, особенно при их недостатке в пище, и является приспособительной реакцией, направленной на поддержание определенного уровня этих веществ в пищеварительных соках.

В значительно меньшей степени изучено выведение в пищеварительный тракт нейтральных жиров (НЖ) и свободных жирных кислот

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SOME EFFECTS OF PHENOLIC ANTI-OXIDANTS ON SODIUM AND POTASSIUM BALANCE IN THE RABBIT

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THIS paper is part of a study of the biological activity of two commercial anti-oxidants, 2 : 6-di-*tert.*-butyl-4-methylphenol (BHT) and butylated hydroxyanisole (BHA), which is a mixture of two isomers, 85 per cent being 2-*tert.*-butyl-4-methoxyphenol and 15 per cent 3-*tert.*-butyl-4-methoxyphenol. These anti-oxidants are used for the stabilization of edible fats. In the course of metabolic studies of BHA (Dacre, Denz and Kennedy, 1956) and BHT (in progress), it was found that a series of daily doses of 1 g. to rabbits led to muscular weakness, inanition and death. Although it is well known that large doses of such phenolic materials are lethal, the mechanism of poisoning has been obscure. It will be shown here that large doses of BHA and BHT produce a gross disturbance of sodium, potassium and water balance in the rabbit.

METHODS

Animals

The rabbits were does of the New Zealand white strain weighing 1·3–2·0 kg. They were maintained on a standard pellet diet (wheat, 40 per cent; pollard, 33 per cent; bran, 27 per cent), and were kept singly in metabolism cages designed to permit the separate collection of urine and faeces. Except where otherwise indicated in the text, water and food were supplied *ad lib.* The daily intake of food and water and the urine volumes were measured. The faeces were generally dry and well-formed and were easily collected and weighed.

Materials and dosage

BHA (Sústane) was obtained from the Universal Oil Products Company, Riverside, Illinois, U.S.A., and BHT from the Shell Company of New Zealand, Ltd. Both BHA and BHT were dissolved in olive oil and 1 g. in 5 ml. of oil was administered by stomach tube to the rabbits each morning. Rabbits usually refused food during the 24 hr. after receiving BHA or BHT. Rabbits given 5 ml. of olive oil also showed a marked reduction in food intake.

Histology

Frozen sections of the adrenal were cut at 10 μ and stained with Oil Red O. Paraffin sections of tissues were stained with Harris's haematoxylin and eosin, Crossman's trichrome and Heidenhain's azan.

Sodium and potassium estimations

All estimations of Na and K were made by flame photometry.

In urine.—Direct determinations were made on appropriate dilutions of urine.

In serum.—Blood was collected from the marginal vein of the ear into silicone-coated tubes. The serum was separated by centrifuging the blood immediately after collection (cf. Cardin and Llaurado, 1955).

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In food and faeces.—About 1 g. of the material was weighed into a micro-Kjeldahl flask and digested with redistilled HNO_3 until the residue was colourless. This residue was taken up in 10 ml. of 0·1 N-HCl and the Na and K determined directly.

In tissues.—Rabbits were killed in a gas chamber and the muscles (tibialis anticus, soleus, biceps femoris), the diaphragm, heart and brain were dissected and samples with a wet weight of 0·3–1·0 g. were taken into weighed stoppered tubes. After reweighing, the tubes were placed in a deep-freeze cabinet for 2–4 hr. The tissues were dried from the frozen state. They were then weighed to give dry weight and the fat was extracted by shaking with 10 ml. of dry ether (dried by distillation from conc. H_2SO_4). A constant weight for extracted tissue was obtained for the biceps femoris, soleus, tibialis anticus and heart after one extraction, diaphragm after two and brain after three consecutive extractions with dry ether. The dry tissue was dissolved in 5 ml. concentrated HNO_3 (redistilled) and transferred to a micro-Kjeldahl flask and digested until the residue was white. The residue was taken up in 10 ml. of 0·1N-HCl for Na and K determinations.

Aldosterone assays

Urine produced by 3–4 rabbits in 24 hr. was pooled and 40 min. after acidification to pH 1 extracted 4 times with chloroform and then re-extracted 4 more times after 24 hr. The extract was evaporated to dryness at reduced pressure, taken up in a small volume of chloroform, dried in a stream of nitrogen, dissolved in 20 per cent ethanol in water and the concentrate assayed on adrenalectomized rats. The rats used in the bio-assay were injected with a portion of the extract and a load of Na and K. The activity of aldosterone was measured by the reduction of the Na/K ratio in the urine of the adrenalectomized rats and expressed as a percentage of the Na/K of a control group injected with solvent alone and given the same load of Na and K on the same day. The figures obtained as percentages have been converted to μg. of aldosterone by a calibration curve obtained previously with pure aldosterone. This method has been fully described by Llaurado (1956*a*, *b*).

Extracellular fluid volume

The thiocyanate method was used. The rabbit's bladder was emptied by suprapubic pressure. A 2 ml. sample of blood was taken from the left ear into a siliconed tube. A dose of 25 mg. of sodium thiocyanate in 2·5 ml. of water was injected slowly into the marginal vein of the right ear. Two hours later the rabbit's bladder was again emptied and a second sample of 2 ml. of blood taken from the left ear. Protein-free filtrates were obtained from the separated serum with 10 per cent trichloracetic acid. The thiocyanate in the protein-free filtrates of serum and in the urine were determined by measuring in a spectrophotometer the intensity of colour developed with ferric nitrate (see Elkinton and Taffel, 1942).

The extracellular fluid volumes are expressed as ml. Rabbits given BHA or BHT lose weight and this loss obscures the fall in extracellular fluid volume that occurs during treatment when the results are expressed as a percentage of body weight. Rabbits with similar initial weight (1·30–1·35 kg.) were chosen for this experiment. The mean value of the extracellular fluid volume of rabbits before dosing with BHA was 416 ml. or 31·5 per cent of body weight.

RESULTS

Increase in urinary sodium and potassium

When daily doses of 1 g. of BHA or BHT are given orally to rabbits there is a ten-fold increase in sodium excretion in the urine. At the same time there is a

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EXPLANATION OF PLATES

FIG. 1.—Z. glomerulosa and fasciculata of normal rabbit adrenal. Stained with Oil Red O.

FIG. 2.—Rabbit adrenal after five doses of BHA showing lipoid depletion in Z. glomerulosa. Stained with Oil Red O.

FIG. 3.—Rabbit adrenal after five doses of BHA. Inner fasciculata showing cells undergoing hydropic degeneration.

FIG. 4.—Kidney from control rabbit showing scarring and fibrosis.

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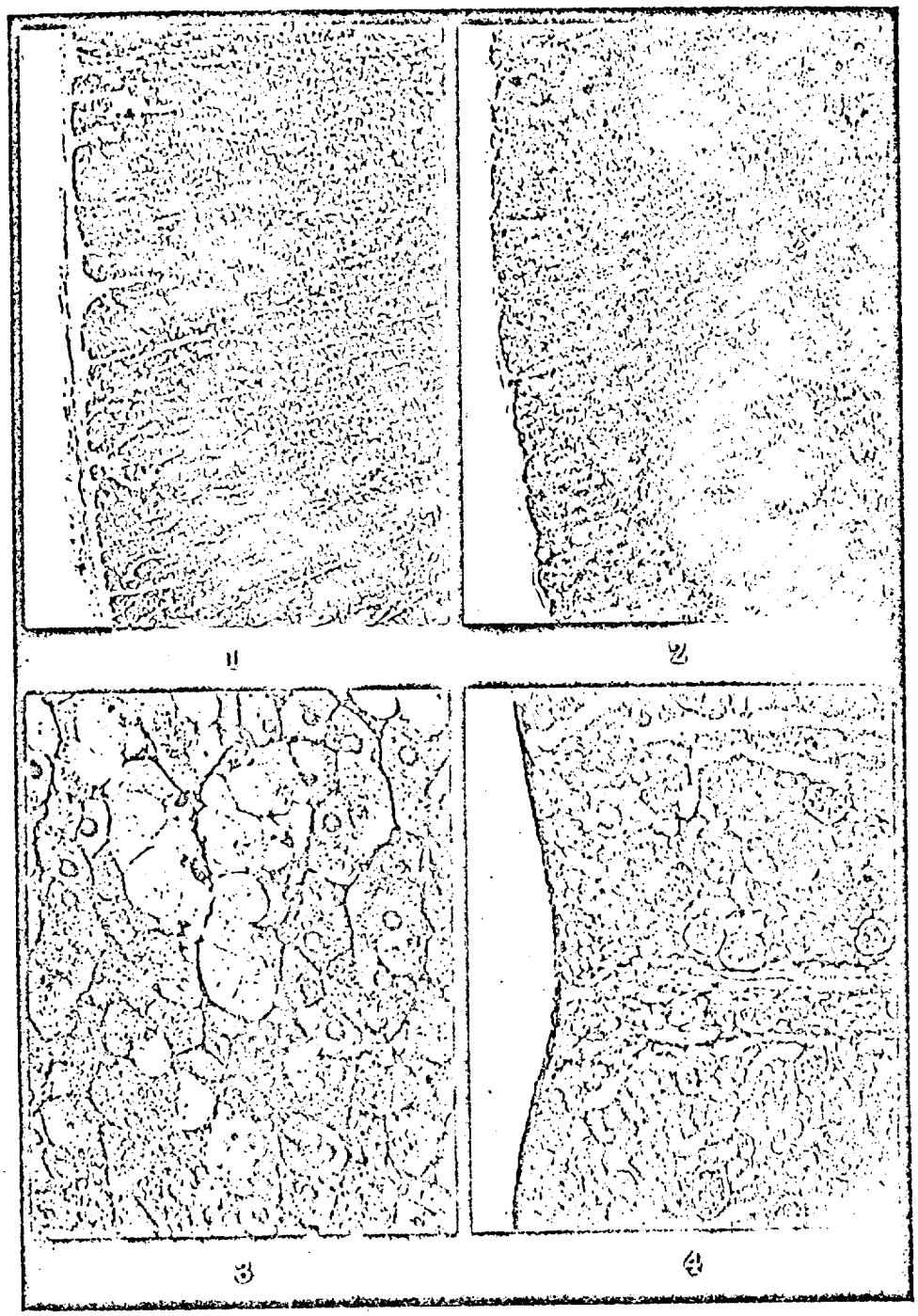

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20 per cent increase in potassium output in the urine. The results obtained with BHA are given in Table I. Similar increases have been obtained with BHT.

TABLE I.—*Effect of Daily Dose of One Gram of BHA on Urinary Sodium and Potassium (as m-equiv./Day)*

	Basal level	Number of doses				
		1	2	3	4	5
Sodium	0·137	0·787	1·179	1·300	0·815	1·063
Potassium	5·394	6·690	5·847	6·058	5·130	5·260

Results are means for 13 animals.

Changes in the adrenal cortex

The normal appearance of the adrenal cortex of the rabbit when stained with Oil Red O is shown in Fig. 1. After a series of three to five doses of BHA or BHT there is a gross depletion of lipoid staining of the zona glomerulosa (Fig. 2). The intensity of staining of the other zones of the adrenal cortex is unaffected. Sections stained with haematoxylin and eosin do not show any cellular necrosis in the zona glomerulosa, but show some increase in cytoplasmic basophilia (cf. Symington and Davidson, 1956).

In sections stained with haematoxylin or by trichrome methods, single cells and small groups of cells in the zona fasciculata and especially in its deeper layers (the inner fasciculata of Nicander, 1952) are seen to be undergoing hydropic degeneration (Fig. 3). In some sections from control animals similar changes are seen occasionally in the zona reticularis but not in the zona fasciculata. In this paper no attempt will be made to evaluate the significance of the changes in the zona fasciculata.

Changes in aldosterone excretion

The remarkable increase in sodium excretion and the loss of lipoid staining in the zona glomerulosa, which seems to be the most likely site of the production of aldosterone (Deane, Shaw and Greep, 1948; Peschel and Race, 1954, and others), showed the need for data on aldosterone excretion in these rabbits.

A biological assay was used. For convenience of exposition the assays are expressed as μg. of aldosterone per day although no great weight is attached to the absolute figures owing to the difficulties that have been found in comparing results obtained in different laboratories (see Dodds, Garrod and Simpson, 1956). The changes in aldosterone level occurring with treatment are regarded as being more significant than the individual figures for aldosterone.

In rabbits treated with BHA or BHT there is a consistent rise in aldosterone excretion (Fig. 5). The average level of aldosterone excretion in normal rabbits was 0·027 μg. per day with a range of 0·003 to 0·061 μg. After dosing with BHA or BHT a high level of aldosterone excretion was maintained for periods up to one week. One group of four rabbits excreted an average per rabbit of 0·044 μg. on the seventh day of treatment compared with an initial level of 0·017 μg. The highest aldosterone levels were reached about the fourth day of treatment (levels of 0·15–0·19 μg.). The later samples showed some decrease in aldosterone but did not fall to pre-injection levels.

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Renal changes

The association of a raised excretion of both sodium and aldosterone suggests that the increased sodium loss is due to changes in renal function. An examination of the kidneys of rabbits from our colony shows that all kidneys are affected by a chronic inflammatory process normally described as pyelonephritis (Fig. 4). The focal lesions of the kidney appear in the rabbits in the first three or four weeks of life before they are weaned. These lesions are sufficiently severe to prevent

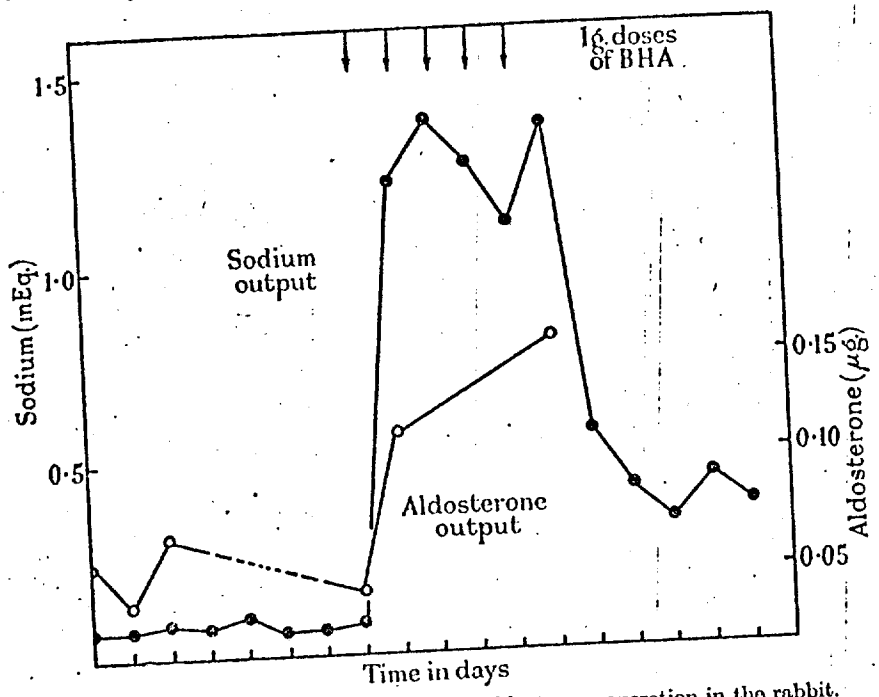

FIG. 5.—Effect of BHA on the Na and aldosterone excretion in the rabbit.

any detailed histochemical studies of the kidneys. In these acute experiments there was no evidence of vacuolation of the tubular epithelium which has been described in chronic potassium deficiency in man and some experimental animals (Conn and Johnson, 1956; Fourman, McCance and Parker, 1956, etc.).

Changes in serum sodium and potassium

The first three doses of BHA or BHT cause a slight rise in serum sodium. Only in the terminal stages is there any fall in the sodium level. Potassium, on the other hand, shows an immediate fall which is progressive and the level falls to about half the normal value after the sixth dose (Table II).

Sodium and potassium balance

The extent of potassium loss in treated animals determined by balance experiments is greater than might be expected from data on urine alone. Figures in Table I show that an increase of 2·01 m-equiv. of potassium above normal levels

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TABLE II.—*Effect of Daily Doses of One Gram BHA on m. equiv./l-Sodium and Potassium in the Serum of Rabbits*

	Basal level	Number of doses 1	2	3	4	5	6	7
Sodium	146	148	150	150	145	145	142	126
Potassium	4·0	3·7	3·2	3·2	2·7	2·7	2·2	1·9

Each figure is the mean of four estimations.

was excreted in six days. This observation fails to take into account the fact that animals dosed with the anti-oxidants in olive oil ceased to eat. This reduces their potassium intake to zero and they are then in negative balance to the extent of the potassium in the urine and faeces. The effect on the potassium balance is shown in Fig. 6, where in the course of a series of six doses of BHA the rabbit

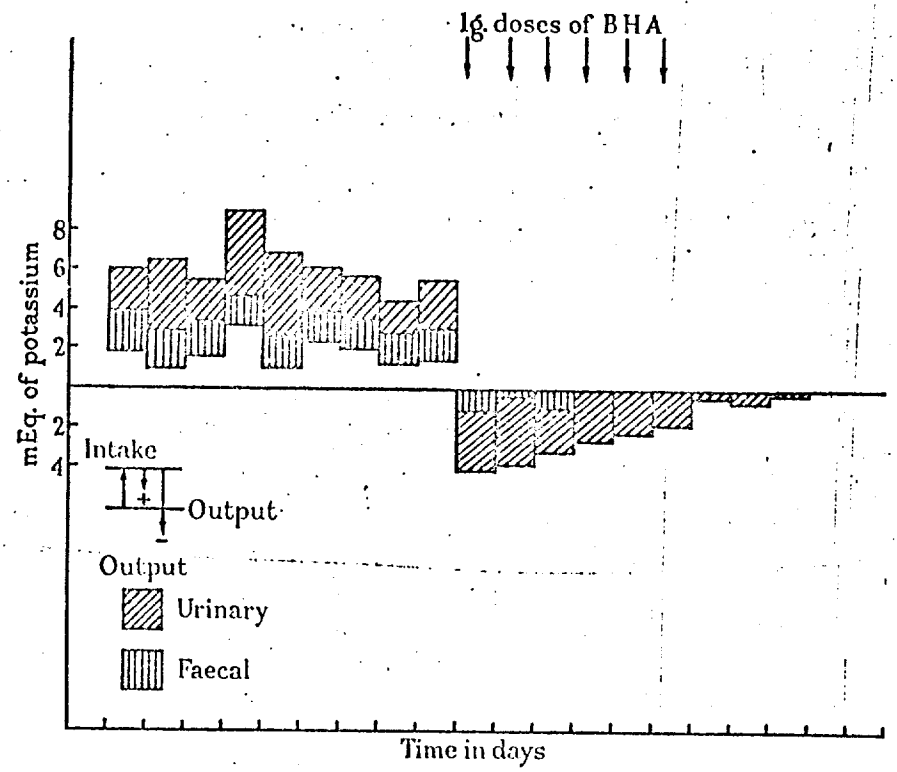

FIG. 6.—Effect of six daily doses of 1 g. of BHA on the K balance in the rabbit. Total K loss 36 m-equiv.

has lost 36 m-equiv. of potassium, which was about half of the potassium normally present in the body. Olive oil alone does not produce any significant potassium loss because the potassium output falls sharply with the decrease in food intake. The effect on sodium balance is less remarkable, and the corresponding sodium loss after 6 doses is 5·7 m-equiv.

The relative proportions of the daily losses of sodium and potassium to the amounts in the extracellular fluids and the cells is shown in Fig. 7. It can be

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seen that the daily loss of sodium is unlikely to produce any marked effect on the sodium in the extracellular fluid, and therefore in the serum, for some days. The daily loss of potassium is grossly in excess of the amount in the extracellular fluid at any one time and can be maintained only by a continual withdrawal of potassium from the cells.

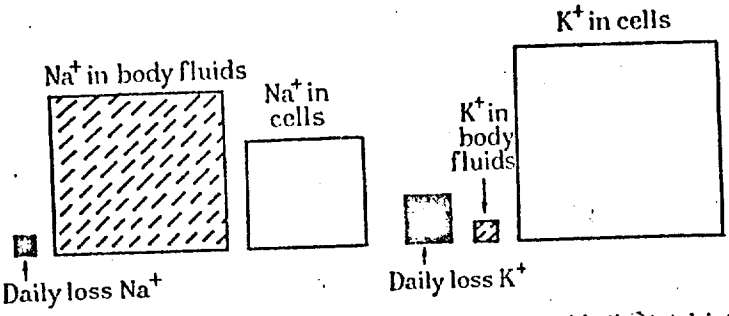

FIG. 7.—Comparison of daily loss of Na and K in treated rabbits with the total intracellular and extracellular Na and K.

Tissue sodium and potassium levels

The data for sodium and potassium are those obtained by direct analysis and no attempt has been made to divide them into extra- and intracellular levels within the tissue. The tissues chosen were white muscle (biceps femoris), red muscle (soleus), mixed red and white muscle (tibialis anticus), diaphragm, heart muscle and brain. These tissues show very different levels (Table III), and they respond differently to the loss of body potassium which occurs when either BHA or BHT is administered. In voluntary muscle, there is a decrease in muscle potassium and an increase in sodium. This change becomes accentuated after four or five days. The changes are shown for the tibialis anticus muscle in Fig. 8. On the other hand, the potassium and sodium levels of brain, heart and diaphragm show very little effect until the terminal stages when, about the sixth day, there is a fall in potassium and a rise in sodium. These effects are shown for heart muscle in Fig. 9. From these results it can be seen that the intracellular potassium is not drawn on uniformly at a time when potassium loss is considerable.

TABLE III.—*Sodium and Potassium in μ. equiv./g. Wet Tissue in Normal Rabbits*

Tissue	Biceps femoris	Soleus	Tibialis anticus	Diaphragm	Heart	Brain
Sodium	15·1±0·7	29·7±1·6	19·6±0·6	25·6±1·3	46·0±3·0	46·8±2·2
Potassium	88·0±4·5	82·2±4·1	94·3±1·2	63·2±2·5	62·9±1·2	71·4±2·5

Means and S.E.M. of 5 estimations.

Cause of death

The profound fall of serum potassium and the reduction in cellular potassium especially in the later stages of poisoning suggests that the rabbits are dying because of potassium deficiency. This conclusion is supported by the observation that the rabbits develop muscular weakness especially in the hind limbs. Rabbits can be kept alive by administering 1 per cent potassium chloride. Some of the

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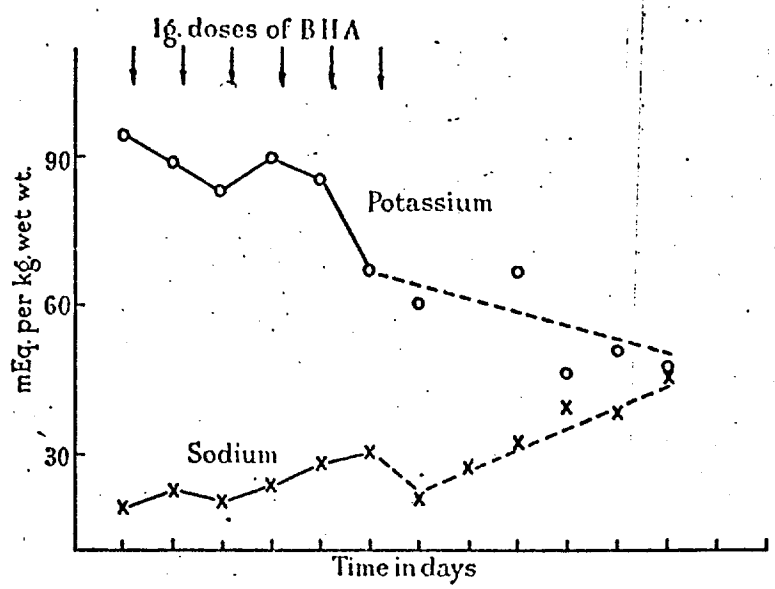

FIG. 8.—Effect of BHA on the Na and K levels in the tibialis anticus muscle. First six points on each curve are means of five animals, remaining points from single animals.

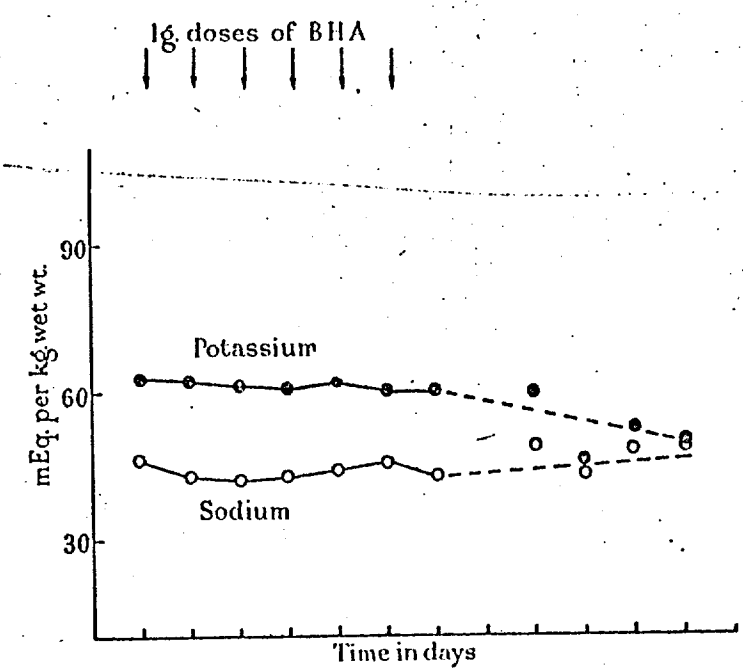

FIG. 9.—Effect of BHA on the Na and K levels in the heart muscle. First six points on each curve are means of five animals, remaining points from single animals.

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affected animals will drink this solution. Others had to receive it by stomach tube.

These findings explain an earlier observation that rabbits which were fed cabbage survived many more doses of BHA or BHT than those supplied only with food cubes. It also explains the failure of sodium chloride therapy which was attempted when the sodium-losing effect was first detected. Replacement therapy with 9-α-fluorohydrocortisone acetate in an attempt to prevent the sodium loss was also tried before the aldosterone assays had been made. In view of the fact that aldosterone excretion was at a high level all the time and that death was due to potassium loss, it was not surprising that corticoid replacement therapy failed to keep the rabbits alive.

Extracellular fluid volume

It has been shown above that in treated animals there is an increased loss of sodium in the urine and an increase in the sodium content of muscle. This happens at a time when the sodium intake has been reduced almost to zero so

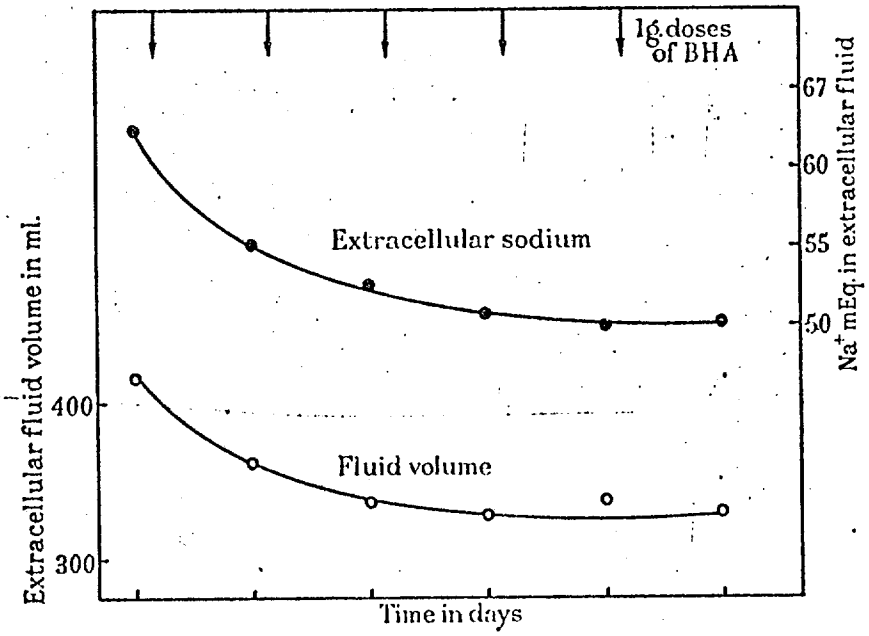

FIG. 10.—Effect of BHA on the extracellular fluid volume and the total extracellular sodium. Each point is a mean of four estimations.

that this movement can only happen at the expense of the sodium in the extracellular fluid. It has been shown that the concentration of sodium in the serum, and hence in the extracellular fluid, does not fall at least for the first five days. These observations are compatible only with a fall in extracellular fluid volume, and this has been found. In Fig. 10 the total amount of extracellular sodium is shown and it will be seen that there is an early fall in the amount of extracellular sodium that continues over the period of five days during which the animals are dosed with the anti-oxidant.

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DISCUSSION

The death of rabbits given 1 g. daily of BHA or BHT is attributed to the loss in the urine of potassium which leads to a fall in the potassium in the muscle and other tissues.

The association of high aldosterone output with excessive potassium loss in man was first reported by Cope and Llaurado (1954), although the significance of this association was not properly understood until Conn (1955) showed that it is due to a hyperfunctional adrenal tumour, and established the clinical syndrome called primary aldosteronism. Subsequently it has been shown (Luetscher and Curtis 1955; Falbriard, Muller, Crabbé and Duckert-Maulbetsch, 1955) that a high aldosterone output can be also associated with excessive sodium loss in the so-called "sodium-losing" nephritis. In this case the primary lesion consists of an inability of the renal tubules to reabsorb sodium, and the hyperproduction of aldosterone is a compensatory mechanism to save body sodium. In the long run, however, this mechanism is detrimental because it may lead to potassium depletion.

In the experiments reported here the association of an increased excretion of sodium in the urine with an increased output of aldosterone suggests that the primary effect of the anti-oxidants is renal and the increased aldosterone secretion is secondary to the sodium loss. The histological picture of selective depletion of lipoid of the zona glomerulosa of the adrenal supports the current view that this is the site of aldosterone production. The relatively high level of aldosterone secretion even in the later stages of poisoning suggests that the loss of lipoid is not evidence of failure of the adrenal, but is a measure of the rapid production of aldosterone.

The underlying cause of the excessive loss of electrolytes in the urine has not been found. Both BHA and BHT are excreted mainly as glucuronides and ethereal sulphates. These materials are comparatively strongly acid and they may carry sodium and potassium with them into the urine. However there is very little increase in ammonium ion in the urine. There is the further possibility that BHA and BHT by their action as anti-oxidants may have a direct effect on the tubular epithelium of the kidney. Preliminary studies of the metabolism of BHT in the rabbit complementary to those already reported on BHA suggest that the metabolism of the compounds is very different. A striking finding in this paper has been the identical effects of these two compounds on the sodium, potassium and water balance in the rabbit.

SUMMARY

Two phenolic anti-oxidants when given to rabbits by stomach tube in doses of 1 g. per day produce similar effects on Na and K excretion in the urine: a ten-fold increase in Na and a 20 per cent increase in K.

The increase in urine Na has little effect on serum Na, and there is an early fall in extracellular fluid volume which parallels a similar fall in the total amount of extracellular Na.

The serum K falls steadily after five days of treatment, and this is associated with a fall in K and a rise of Na in voluntary muscle. Heart muscle shows little change in Na and K levels until the terminal stages.

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The adrenal cortex shows a gross decrease in fat staining of the zona glomerulosa, the region believed to be the site of aldosterone production. At the same time there is an increased excretion of aldosterone in the urine.

A series of daily doses of either anti-oxidant leads to muscular weakness, inanition and death, which is attributed to the loss in the urine of K leading to a fall in the K in the muscle and other tissues.

It is suggested that the primary effect of the phenolic anti-oxidants is renal and that the increased aldosterone secretion is secondary to the Na loss. The underlying cause of the excessive loss of electrolytes in the urine has not been found.

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Abstracts of Papers American Chemical Society - 158th Mtg.
Sep. 7-12 1969

DIVISION OF AGRICULTURAL AND FOOD CHEMISTRY AGFD

40. INHIBITION OF BRADYKININ ACTIVITY BY GALLATES AND OTHER ANTIOXIDANTS. L. F. Fosati, K. K. Fox and M. J. Pallansch. Dairy Products Laboratory, Eastern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture, Washington, D. C. 20250.

In studies of the kinin activity of cow's milk it was observed that antioxidants suppressed the activity of bradykinin used as a control. Gallic acid, its esters and BHA will suppress the contractile response elicited from the smooth muscle of the guinea pig ileum by the action of bradykinin. The initial suppressive effect of members of a homologous series of gallates increases almost directly with chain length. The amyl ester is 150 times as effective as the methyl homolog which requires a concentration of 6.6×10^{-7} gm/ml to start inhibiting the action of 4.0×10^{-8} gm/ml bradykinin. Hexyl gallate is slightly less effective and the higher homologs are too insoluble to produce suppression of bradykinin action in aqueous systems. BHA is the most effective suppressing agent of the antioxidants tested. A plot of the reciprocal of gut response against the reciprocal of bradykinin concentration for different antioxidant concentration indicates that the antioxidants might act as competitive inhibitors. The suppressive effect of long chain gallates, when used in sufficient quantity to markedly depress the gut response to bradykinin, is only partially reversible.

41. SYNTHESIS OF THE FOURTH COMPONENT OF THE BOLL WEEVIL SEX ATTRACTANT, 2-ISOPROPENYL-1-METHYLCYCLOBUTANEETHANOL. J. H. Tumlinson, R. C. Gueldner, D. D. Hardee, A. C. Thompson, P. A. Hedin, Boll Weevil Research Laboratory, Box 5367, State College, Miss., 39762; and J. P. Minyard, Miss. State Chemical Lab., Box CR, State College, Miss. 39762.

The structures of the four compounds (I, II, III, and IV) which comprise the boll weevil sex attractant have been previously reported.

I II III IV

Additionally, the identity of three of these compounds was proved by synthesis. Subsequently, synthesis of the fourth component has been completed, and a synthetic mixture which is equivalent to the natural pheromone in attractancy has been formulated. Synthesis of 2-isopropenyl-1-methylcyclobutaneethanol, was achieved by a four-step synthesis which includes photolysis, Grignard addition, hydroboration, and dehydration. The results of bioassays and field tests will be reported.

42. AN IMPROVED SYNTHESIS OF THE BOLL WEEVIL SEX ATTRACTANT COMPONENTS. R. C. Gueldner, J. H. Tumlinson, D. D. Hardee, A. C. Thompson, P. A. Hedin - Boll Weevil Research Laboratory, Box 5367, State College, Miss. 39762 - and J. P. Minyard, Miss. State Chemical Lab., Box CR, State College, Miss. 39762.

An improved synthesis of the previously reported boll weevil sex attractant components, cis-3,3-dimethyl-$\Delta^{1,\beta}$-cyclohexaneethanol, cis-3,3-dimethyl-$\Delta^{1,\alpha}$-cyclohexaneacetaldehyde and trans-3,3-dimethyl-$\Delta^{1,\alpha}$-cyclohexaneacetaldehyde, has been accomplished. The synthesis provides high yields of the two desired olefinic isomeric precursors and allows their separation by distillation. The elucidation of the stereochemistry of the isomeric precursors by NMR spectra will be discussed. The synthesis of analogs and structure-activity relationships will also be presented.

43. THE APPLICATION OF THE TRICHLOROACETYL ISOCYANATE REACTION WITH TERPENE ALCOHOLS FOR THE QUANTITATIVE FRACTIONATION OF ESSENTIAL OILS. P. A. Hedin, R. C. Gueldner, and A. C. Thompson, USDA Boll Weevil Research Laboratory, Box 5367, State College, Miss. 39762.

The tertiary alcohols of essential oils are often difficult to separate from other oxygenated classes by existing techniques. Previously, Coodlett reported the employment of trichloroacetyl isocyanate (TCAIC) to esterify hydroxy compounds for characterization of alcohols by nuclear magnetic resonance. As applied to gas-liquid chromatographic (GLC) profile analysis of oils, injection of the TCAIC reaction mixture gave a reduction in peak numbers; however removal of the TCAIC esters from the oil with subsequent regeneration to the original alcohol would provide additional valuable information. This report describes the development of a quantitative procedure for the solvent extraction of terpene alcohol esters from the essential oil with their concomitant hydrolysis to carbamates, and the conditions for quantitative regeneration (saponification) of the alcohols.

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adjacent profiles indicate that the interpretation given is reliable. Full utilization of the reflection data will probably increase the precision of the interpretations somewhat. Table 1 shows the pertinent data for eight similar crustal sections that were obtained during the cruise. [See (*2*) for parts of the geologic column not of primary interest here and for details of the analysis techniques and results.]

As indicated in Table 1, mantle refractions were clearly obtained with the air gun source. A few arrivals were verified by the use of explosives and conventional records (*1, 2*).

The somewhat irregular nature of the refraction arrival line suggests that the interface either is irregular or consists of a thin zone of subordinate interfaces rather than a sharply defined plane.

The observations were taken along a thousand-kilometer-long line running roughly northeast from near the Marshall Islands to southwest of the Hawaiian Islands in water depths averaging over 5 km (*2*). The extent and deep-ocean environment of these observations suggest that the 6.9 to 7.6 km/sec (average, 7.3 km/sec) crustal layer may be fairly widespread throughout the south-central Pacific Ocean. Although similar velocity values have been reported from such oceanic environments as the Caribbean Sea (*3*) and, in the Pacific Ocean, the Shatsky Rise (*4*) and the Hawaiian Arch and Deep (*5*), the layer has not been a generally accepted feature of classical deep-ocean basin structure. For example, Ewing, Raitt, and Shor and Raitt (*6*) in their reviews of the literature indicate that the deepest oceanic crustal layer has a characteristic velocity of around 6.8 km/sec in the great majority of cases.

If the 7.3 km/sec layer is widely present, as our measurements suggest (*7*), the implications are several, such as: (i) a widespread change in composition or a polymorphic transition at this interface; (ii) a higher than heretofore accepted mean density of the earth's crust affecting depths determined from gravity anomalies and perhaps density distributions consistent with the moment of inertia; (iii) an effect on the dispersion of seismic surface waves requiring a reconsideration of current interpretations.

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References and Notes

1. This layer was first reported by G. H. Sutton, G. L. Maynard, D. M. Hussong, in paper 15, presented at the April 1969 annual meeting of the Seismological Society of America, St. Louis, Mo. (April 1969); it was discussed further by G. L. Maynard, G. H. Sutton, D. M. Hussong, in paper O132, presented at the annual meeting of the American Geophysical Union, Washington, D.C. (April 1969) [see also *Trans. Amer. Geophys. Union* **50**, 206 (1969)].
2. G. L. Maynard, G. H. Sutton, D. M. Hussong, two papers in preparation.
3. C. B. Officer, J. I. Ewing, J. F. Hennion, D. G. Harkrider, D. E. Miller, *Phys. Chem. Earth* **3**, 17 (1959); H. W. Menard, *J. Geophys. Res.* **72**, 3061 (1967); J. I. Ewing, J. Antoine, M. Ewing, *J. Geophys. Res.* **65**, 4087 (1960). A layer with refraction velocity of 7.0 to 7.5 km/sec is a well-known feature of the "mid-ocean" ridges, certain oceanic margin areas, and some trenches and their associated ridges. See, for example, B. C. Heezen and M. Ewing, in *The Sea*, M. N. Hill, Ed. (Interscience, New York, 1963), vol. 3, p. 388; X. Le Pichon, R. E. Houtz, C. L. Drake, J. E. Nafe, *J. Geophys. Res.* **70**, 319 (1965); M. Talwani, G. H. Sutton, J. W. Worzel, *ibid.* **64**, 1545 (1959); R. E. Houtz and J. I. Ewing, *Bull. Seismol. Soc. Amer.* **54**, 867 (1964); E. T. Bunce, D. A. Fahlquist, J. W. Clough, *J. Geophys. Res.* **74**, 3082 (1969).
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7. More recent work reveals that the 7.5 km/sec layer is not confined to the area considered here: D. M. Hussong (personal communication) finds the layer in the deep ocean north of Hawaii. D. M. Hussong, A. A. Nowroozi, M. E. Odegard, and G. H. Sutton reported the layer 180 km west of San Francisco in paper S16, presented at the fall meeting of the American Geophysical Union, San Francisco (December 1969) [see also *Trans. Amer. Geophys. Union* **50**, 644 (1969)].
8. This work was supported by the National Science Foundation under grant GA 1639 and by the Office of Naval Research under contract Nonr 3748(05). Hawaii Institute of Geophysics Contribution No. 317.

11 December 1969

Bradykinin Inhibition by Butylated Hydroxyanisole

Abstract. *Concentrations of butylated hydroxyanisole as low as 8×10^{-9} mole per liter can inhibit detectably the contraction of smooth muscle elicited by bradykinin. The mechanism of the inhibitory effect of this food grade anti-oxidant is apparently complex, and the effect is only partially reversible.*

Bradykinin's contractive action on smooth muscle is inhibited by agents generally classed as tranquilizers (*1*). We now report similar inhibition of bradykinin activity by butylated hydroxyanisole (BHA), an antioxidant widely used for the preservation of foods containing unsaturated lipids.

Assays of this inhibitory effect were made with a 2- to 3-cm section of the terminal segment of a guinea pig ileum suspended in a 5-ml capacity perfusion bath held at 37°C. Changes in length of the ileum were recorded with a standard kymograph. The gut was equilibrated against Tyrode's buffer containing 2 mg of atropine and 40 μg of pyribenzamine per liter by flowing the solution through the cell at a rate of 15 ml per minute for 1 hour before use.

After equilibration the flow of Tyrode's buffer through the cell was suspended, and a 0.2-ml portion of test solution containing pure bradykinin and the antioxidant were injected into the cell. Test solutions were made from stock solutions of butylated hydroxyanisole (Nutritional Biochemicals) dissolved in water and synthetic bradykinin (BRS 640, Sandoz) dissolved in Tyrode's buffer. After exposure for 30 seconds to a test solution, the mixture was flushed from the cell, and the entire procedure was repeated with another kinin mixture. The response of the gut to a solution containing only pure kinin was used throughout the analyses as a control. The height of the peak traced on the kymograph chart when the gut was in contact with the kinin-containing solution was taken as

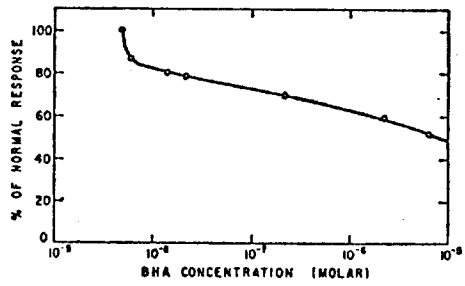

Fig. 1. Suppression of ileum response to test solutions of bradykinin containing increasing amounts of BHA. Gut was freed of reaction mixtures by flushing Tyrode's buffer through the cell at a rate of 15 ml/min for 10 minutes between each point determination. The raw data were corrected for irreversible decreases in gut sensitivity due to exposure to BHA.

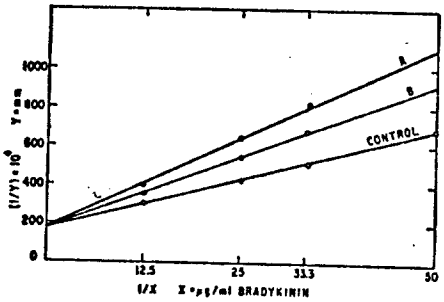

Fig. 2. Drug dose (x)–response (y) plot characterizing suppression of contractile response of smooth muscle (guinea pig ileum) by BHA. Control samples contain no antioxidant. Points on line A were obtained with 7.2×10^{-9} g of BHA per milliliter in test solution, and points on line B were obtained with 3.6×10^{-9} g of BHA per milliliter. All points represent data corrected for loss in gut sensitivity to bradykinin due to exposure to BHA.

being directly proportional to the amount of smooth muscle contraction elicited by bradykinin. Reduction in peak height by added antioxidant was taken as a measure of its inhibitory action.

With this procedure, it was found that the threshold concentration at which BHA began to suppress the ileum's response to 0.04 μg of bradykinin per milliliter in the reaction bath was $8 \times 10^{-9}M$. Increasing concentrations of antioxidant depressed gut response (Fig. 1).

Supposedly the mechanism of this inhibition can be ascertained from a standard study of the relation of drug to dose response as described by Roche e Silva (*2*). Here the reciprocals of effects ($1/Y$), where Y is peak height in millimeters, as measured on the kymograph chart are plotted against the reciprocals of the doses ($1/X$), where X is equal to the concentration of bradykinin in the reaction chamber. Our data in this form are presented in Fig. 2. From the relatively straight line plots and the common intercept shown in Fig. 2 it can be inferred (*2*) that BHA acts as a competitive inhibitor of bradykinin. Likewise, from the second plot, the relative inhibitor strength of BHA can be derived (*2*) as its inhibitory potency (pK_1). The value ascertained by this procedure, $pK_1 = 7.9$ g/ml, indicates that BHA is a relatively strong inhibitor.

The validity of this relation may be questioned since the inhibitory action of BHA is not completely reversible. After four 30-second exposures to solutions containing $1 \times 10^{-6}M$ BHA stock solution, only 92 percent of normal bradykinin response could be recovered by flushing with buffer for 15 minutes. The reason for this loss in sensitivity has not been established. It probably involves very strong or irreversible binding of the antioxidant on some bradykinin receptor sites since the smooth muscle contracts normally in response to 0.40 μg of histamine per milliliter in the presence of $10^{-5}M$ concentrations of BHA in the reaction bath. Regardless, our data demonstrate that relatively low concentrations of BHA can inhibit the contractile action of bradykinin on smooth muscle. The higher BHA concentrations used in these experiments approach the concentrations of BHA permitted in foods under present Food and Drug Administration regulations. The significance of this finding in regard to the widespread use of this antioxidant in food manufacture remains to be established.

The possibility that an impurity in the commercial BHA sample used in our study could have given rise to the described results has been considered. Standard gas-liquid chromatographic analysis of the antioxidant used showed it to be essentially 7 percent 2-BHA and 93 percent 3-BHA. Only two volatile contaminants in trace quantities could be found on the chromatogram. A sample of BHA further purified by sublimation also has the same inhibitory power as the commercial sample. Furthermore, we have some data that indicate other phenolic antioxidants similarly inhibit the response of smooth muscle to bradykinin.

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Catch Property in Single Mammalian Motor Units

Abstract. *The tension output of single motor units in the cat triceps surae muscle was studied during patterned stimulation of the innervating motoneurons. With repetitive stimulation within a rather narrow frequency range, the tension output of slow-twitch (type S) motor units may be quite sensitive to the pattern of stimulus intervals in the train. The presence of only one stimulus interval that is much shorter than the others in the train can cause marked, long-lasting tension enhancement, provided that the stimulus repetition rate in the basic train is within certain limits.*

A motor unit in skeletal muscle consists of a neural component, a motoneuron with cell body in the spinal cord and axon in a particular muscle nerve, and a muscle component, the bundle of muscle fibers innervated by that motoneuron (*1*). Motor units may be regarded as the indivisible functional elements of skeletal muscle action. Much work on the production and maintenance of muscle tension has involved whole muscle, with all motor units activated simultaneously (*2*). However, such simultaneous activation is rare in normal movement; usually, muscle tension is produced by motor units acting asynchronously and independently (*3*). Moreover, motor units in a given muscle may differ from one another in contractile properties (*4*). For these reasons, we have recently begun a study of the production and maintenance of tension by single motor units in the cat triceps surae muscle. In the course of this work we have observed a phenomenon similar to what has been termed the "catch property" of certain invertebrate muscles (*5*).

When a single motoneuron can be stimulated in isolation, the mechanical response produced in a muscle by one active motor unit can be studied (*4*). In these experiments adult cats, anesthetized with pentobarbital, were maintained with normal blood pressure and body temperature. The tendons of the three heads of the triceps surae muscle, medial gastrocnemius (MG), lateral gastrocnemius (LG), and soleus (SOL), were separated from one another and arranged for independent connection to a strain gauge myograph through a stiff link. The hind limb was rigidly fixed to a frame with drills through the femur and ankle. The strain gauge, mounted on a rack and pinion that permitted

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Micro-invasion of Epidermis caused by Substituted Anisoles

ONE of the earliest morphological lesions to be observed in tumours of the epidermis is the protrusion of pseudopods through defective areas in the basement membrane—a phenomenon termed micro-invasion[1]. Such lesions have been found in the early stages of experimental tumours induced by the local application of the carcinogen dimethylbenzanthracene to the cheek pouch epithelium of hamsters, whereas non-carcinogenic irritants failed to produce them[2]. It therefore seems from these findings that micro-invasion may serve as an early sign of a neoplastic change in the epithelial cells and hence might be interpreted as an indication of the carcinogenicity of substances which produce it. Since our initial report of basal cell pseudopods caused by applications of 4-hydroxyanisole and 3-hydroxyanisole to guinea-pig epidermis[3], we have investigated a number of related substances (Fig. 1). Some of these, notably 7C-phenyl-4-hydroxyanisole and butylated hydroxyanisole (BHA), have been shown to cause micro-invasion.

The experiments were performed on an inbred strain of black guinea-pigs. Applications of the substances made

Fig. 2. Micro-invasion in phenyl hydroxyanisole-treated skin showing basal cell pseudopod (P) invading the upper dermis (D) through the basement membrane (↑).

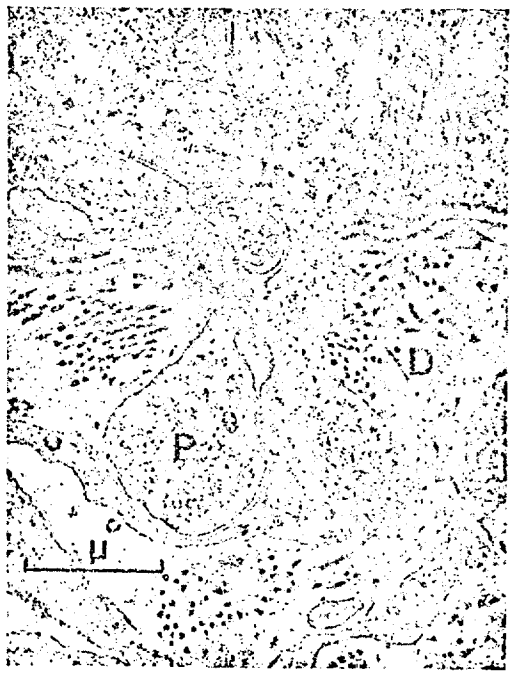

Fig. 3. Micro-invasion in BHA-treated skin.

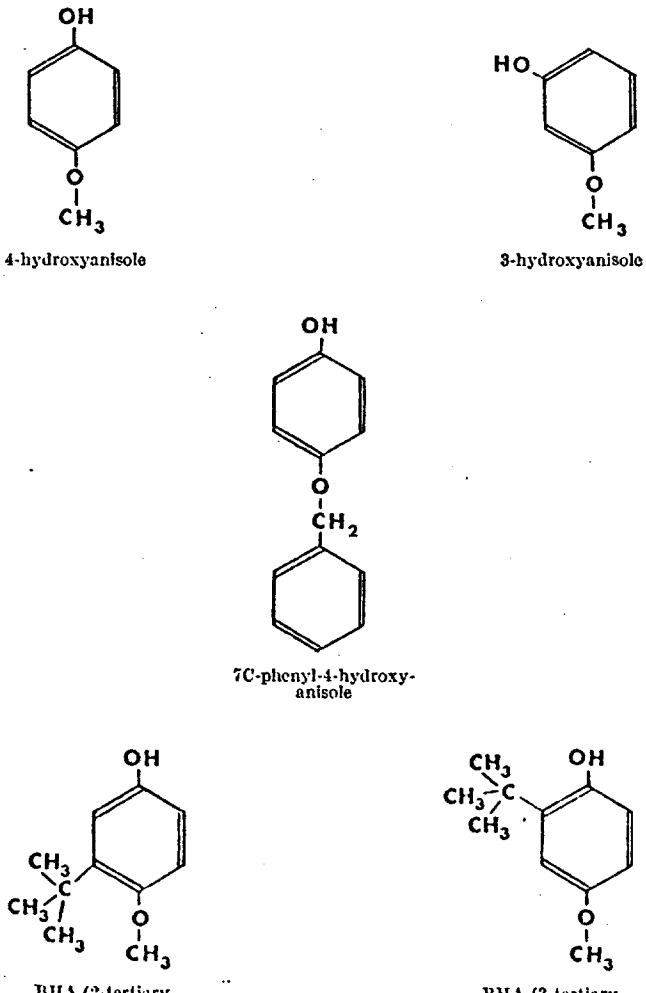

Fig. 1. Structural formulae of substituted anisoles.

up in concentrations of 20 per cent in lanolin were made to the ears of the animals once daily for periods up to 6 weeks. Biopsies were taken at weekly intervals and the material fixed in phosphate buffered 2 per cent osmic acid and embedded in 'Araldite'. Sections stained with uranyl acetate in methanol and aqueous lead citrate were examined in a Siemen's Elmiskop 1 electron microscope.

Micro-invasion by basal cell pseudopods was observed in material from 2 weeks' treatment onwards and there was an associated destruction of the superficial connective tissue with fragmentation of the collagen (Figs. 2 and 3). No such changes were observed in control material from animals either untreated or treated with lanolin alone.

It is of considerable interest that hydroxyanisoles as a group can induce micro-invasion, in view of the demonstrated association between micro-invasion and neoplastic change in epithelial cells.

The result with BHA is particularly significant because butylated hydroxyanisole is used extensively as an anti-oxidant in foodstuffs, although it must be pointed out that the concentrations used in these experiments greatly exceed the concentrations of anti-oxidant present in foods. It does not necessarily follow that the effect of BHA in chronic low dosage would be the same as it is in the high dosage used in these experiments. Nevertheless the data on the possible carcinogenicity of BHA are insufficient to exonerate it[1].

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Primary Structure Homology within the 23S Ribosomal RNA

OUR understanding of the evolution of the cell will depend largely on our gaining an understanding of the evolution of the cell's translation apparatus, and in this context I wish to discuss the recent finding that the RNA sequences (seen in a T1 ribonuclease digest) surrounding the methylated bases in the *Escherichia coli* 23*S* ribosomal RNA (*r*RNA) are all repeated twice in the molecule[1]. This has been interpreted to mean either that the 23*S* *r*RNA molecule is actually a dimer of two identical halves, or that the 23*S* cistron has evolved through a gene duplication and joining mechanism[1]. The dimer interpretation seems to be ruled out by the fact that there are many more kinds of sequences in the 23*S* *r*RNA than there are in the 16*S* *r*RNA, and so, reasonably, the former should be larger than the latter, if most sequences were singly represented in each[2]. In view of the evolutionary significance of the second possibility, however, I wish to point out that there is at least one other reasonable interpretation of these data (which does not invoke gene duplication) and so they cannot be taken as evidence for an origin of the 23*S* *r*RNA cistron by a duplication and joining involving a smaller cistron.

It seems reasonable to assume that the ribosome contains two sites for *t*RNA binding[3] which are nearly identical in composition, and that the methylated bases reside in (and are essential to the function of) these sites. If these assumptions are valid then the twice repeated segments in 23*S* *r*RNA could have evolved as a result of identical functional constraints operating in their evolution—in other words, this would be a case of "convergent evolution" not "divergence" from a common ancestor.

An additional point that tends to reinforce this alternative interpretation is that the introduction of odd bases into RNA requires information, undoubtedly in the form of RNA sequence in the vicinity of the base to be altered, which could put additional pressure for constancy on those sequences surrounding such bases.

I should point out, however, that certain other evidence actually does suggest an origin of the 23*S* *r*RNA cistron by a gene duplication mechanism. I refer to the reports that in *E. coli* there exists primary structural homology between the 16*S* and 23*S* *r*RNAs, seen in the complete competition between 16*S* and 23*S* *r*RNAs for hybridization with *E. coli* DNA[4,5]. If true, this may mean that 23*S* *r*RNA comprises two halves, each homologous to the entire 16*S* *r*RNA molecule, and so each half homologous with the other. The study, showing the methylated base-containing segments in the 23*S* *r*RNA to be two-fold redundant[1], revealed no similarity between those segments in the 16*S* *r*RNA molecule and their counterparts in the 23*S* molecule, however, so it is still reasonable that these particular redundancies came about through a "convergent" evolution.

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Effect of Actinomycin D on the Assimilation of a Cytoplasmic Determinant in Amoebae

CERTAIN cellular properties of the large free living amoebae, *Amoeba proteus* and *Amoeba discoides*, such as sensitivity to streptomycin and nuclear diameter, have been shown to be influenced by self-replicating cytoplasmic hereditary determinants[1]. *A. proteus* is more sensitive to streptomycin and has a greater nuclear diameter than *A. discoides*[2,3]. Micro-injection of small quantities of cytoplasm of *A. discoides* into recipient *A. proteus* results in the transfer and expression of the determinant studied—for example, lowered sensitivity to streptomycin—for a small fraction of the clones derived from cells surviving the operation[1].

The factors which influence the assimilation and presumably replication of non-homologous cytoplasm are unknown. The small percentage of altered clones, comparable with the small percentage of transformants obtained in bacterial studies[4], could be a consequence, for example, either of a small chance of sampling the presumed randomly distributed determinant, or of the reaction of the recipient cell to "foreign" cytoplasm.

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BIOCHEMISTRY

Leukemia in Mice and Characteristics of Its Development During the Action of Inhibitors from Chain Oxidizing Processes

N. M. Emanuel' and L. P. Lipchina

(Presented by Academician N. N. Semenov 6 V 1958 (May 6, 1958).

The progressive development of a malignant tumoral process finds a great deal of analogy in the course of transient chemical processes, for example, branching chain reactions, discovered by N. N. Semenov (1). The possibility has not been excluded that, on the basis of analogies observed, there lies a similarity in the nature of active, intermediate substances, which are responsible for the self-accelerated character of development of processes in vivo and in vitro. Free radicals represent the active centers of chain reactions. In slowly developing (not explosive) branching chain reactions, there are formed, in addition to this, intermediate substances, which occasionally decompose with the formation of free radicals; as a result of this, there develop degenerated (delayed) branchings, and the process slowly self-accelerates in the course of time.

There exist fully determined indications that in the mechanisms of some biochemical processes a part is played by free radicals by chain reactions (2). It is particularly essential that, from this point of view, an examination be made of reactions of biological (enzymatic) oxidation, which exactly yield the required energy for the growth of malignant neoplasms.

Also, one cannot help giving consideration to the fact that, in many cases, the mechanism of carcinogenesis may be conceived as proceeding from the possibility of the appearance, in the biological substrate, of free radicals,

and of the stimulation, by them, of radical (chain) reactions (3).

A number of facts may serve as a confirmation of this opinion. The action of penetrating radiations on the living organism, which result, in the final analysis, in the formation of free radicals and atoms, is capable of bringing about a radiation cancer and leukemia. Carcinogenic (polycyclic) hydrocarbons markedly stimulate various biological effects, which proceed under the influence of light (photodynamic effect). The sensitizing action of carcinogens is examined as a result of their passage during the absorption of a light quantum in the triplet (biradical) state; this is found to be in conformity with the general theory involved in the action of photosensitizers associated with A. N. Terenin (4). It is customary to assume that, under suitable conditions, the formation of free radicals may take place even in the absence of light (in the case of carcinogen administration), as a result of the interaction of a carcinogenic hydrocarbon with a suitable substrate. The dependence of the frequency of affection with skin cancer on the intensity of the action of even solar radiation makes it possible to point to the development, in the epidermis, of photo-chemical radical reactions, which stimulate a pathological process.

The concept concerned with the freely radical mechanism of carcinogenesis and with the development of a pathological process, and also, with the analogy with chain, degenerative-branching reactions, naturally leads to a conclusion regarding the feasibility of attempts of influencing the growth of malignant neoplasms by means of the introduction, into the organism, of substances that readily interact with free radicals (that break the chain).

Biological oxidation represents the fundamental source of energy for the synthesis of large masses of albumin (protein). For this reason, for the number of multiplying, growing, and in a number of cases, also differentiating cells, G, one may write down the relationship:

G/ is approximately DELTA F is approximately INTEGRAL $omega_{oxidation}$ dt, (1)

where $ometa_{oxidation}$ represents the rate of biological oxidation, which is stimulated (according to the hypothesis) by the inclusion of radical reactions.

((Series of graphs on pg. 142 of text)) ((to the left of the series on the left)): number of cellular forms, in 1 cubic millimeter x $10^{minus\ 3}$; ((in the lower part of the series of graphs on the left, reading down and across)): 8 milligrams; 16 milligrams; 40 milligrams; 8 milligrams; 40 milligrams; 16 milligrams; ((in the lower right-hand corner of the same series)): days; ((underneath this series)): Fig. 1. Dynamics of the growth of the overall amount of leucocytes (black dots) and of immature cells (small circles) in 1 cubic millimeter of blood in 6-week mice of the afb line. Control (1, 2) and experiment in the presence action on the part of the preparation (II) (3, 4). The moments of preparation administration are shown by means of arrows.

((to the left of the graphs on the right)): Number of leucocytes in 1 mm^3 x $10^{minus\ 3}$; of hemocytoblasts in 1 mm^3 x $10^{minus\ 3}$; ((in the lower right-hand corner of these graphs)): days; ((underneath the same graphs)): Fig. 2. Dynamics involved in the content change of the leucocytes (black dots) and in that of the immature cells (small circules) in 1 mm^3 of blood in two recovered 8-week mice of the afb line as a result of the action of the preparation (1). The moments of administration of the preparation are shown by means of arrows in an amount of 3 milligrams in the injection.

((text continued)): In the capacity of substances containing reactive groups, which react with free radicals, we decided to test strong, nontoxic inhibitors associated with chain oxidative reactions (food antioxidants): (I) butyloxyanisol (a mixture of two isomers: 2- and 3-tertiary butyl-4-oxyanisols); (II) ionol (2,6-di-tertiary butyl-4-methylphenol); (III) propylgallate (normal propyl gallate) and others. The presence, in these compounds,

of strong orientants of the first order in ortho- and para-positions, and their screening action determine a high reaction capacity of hydrogen belonging to the hydroxyl group. For the purpose of studying the action of inhibitors, associated with chain oxidizing processes, on the duration and outcome of a tumoral disease, we made use of transplantable, acutely developing leukemias in highly-leukemic mice of the afb line, strain LIV, and in practically non-leukemic black mice of the C_{57} line, strain LA, obtained in the transplantation of leukemia produced in mice of the C_{57} line by means of X-rays.

Transplantable leukemia represents a convenient experimental tumoral model; hematological examinations make it possible to trace the course of the tumoral process in accordance with the quantitative and qualitative shifts of the cellular shapes.

Experiments were carried out on mice having reached a 6-8-week age, with a weight ranging from 15 to 23 grams. The transplantation was carried out intraperitoneally, in accordance with the customarily accepted procedure that assures 100% leukemia transplantability and 100% death of the mice. 6-week mice (15 - 17 grams) died on the 5 - 6th day following the transplantation, and the 8-week ones (18 - 23 grams) -- on the 8 - 10th day). Altogether under experiment were 198 inbred mice. In therapeutic experiments on 8-week mice of the afb line (breed), there were 70 (36 experimental, and 34 control); of mice belonging to the C_{57} line -- 48 (24 experimental, and 24 control); the remaining mice (6 - 8 week) were utilized for the selection of dosages and for studying the biological action of the preparations. ((Three illustrations on pg. facing pg. 42 of text)) ((underneath these illustrations, in Russian letters): a; b; v; ((underneath the same illustrations)): Fig. 3. Picture of the medulla ossium (bone marrow) in mice belonging to the C_{57} line; a -- control, untreated mouse; b -- mouse that had received preparation (III); v --

healthy mouse. Radiation immunity, 60; approximately 10 X. Zeiss microscope. ((text continued)): The administration of the preparations started from the 2nd day following the transplantation. Preparations (I) and (II) were injected hypodermically 3 - 10 times; preparation (III) was administered intraperitoneally in the course of 5 days. Preparations (I), (II) were diluted in apricot oil, and preparation (III) -- in a physiological solution. Preparation (I) was administered figuring on 150 milligrams/kilogram in each injection; (II) -- 200 milligrams/kilogram; (III) -- 37.5 milligrams/kilogram. The control mice were injected with corresponding amounts of a solvent. In some experiments, single doses of preparations (I) and (II) were increased by 2- 10 times. In the case of large dosages in mice weighing 15 - 17 grams, there were noted a drop in weight and symptoms of dyspepsia; larger mice showed good endurance to high doses of the preparation. The chemotherapeutic index (DL : DPO) for preparation (I) is equal to 20, and for preparation (II), 30.

In healthy mice, both in the case of a single administration of 30 milligrams of preparation (I) and 80 milligrams of preparation (II), as well as in the case of a chronic administration over a period of 2 months, 3 - 8 milligrams of preparations (I) and (II), drops in weight and symptoms of dyspepsia were not observed, the blood and the bone marrow remained within the normal range, and no pathological changes, on the part of the internal organs, were observed.

In Fig. 1 is shown an example of growth in the overall number of leucocytes and individual, immature cells (hemocytoblasts and lymphoblasts) in the peripheral blood, in the case of a transplanted leukemia in mice belonging to the afb line, without administrating any preparation (control), and in the case of triple injections of preparation (II) figuring on 8 - 40 milligrams to a mouse for one injection. In this experiment were utilized 6-week

mice (15 - 16 grams) in which, as has already been indicated, leukemia was developing exceptionally acutely. For this reason, the data obtained should be examined only from the point of view of evaluating the effect of the preparation on the dynamics of the hemotalogical changes. There is a clear visualization of the inhibition in the process of growth both in the overall number of leucocytes, as well as in that of immature cells. The growth of the number of immature cells (hemocytoblasts) is described by the formula:

$$G \text{ is approximately equal to } f_1(\text{phi})\, e^{\text{phi } t}; \qquad (2)$$

this is mathematically identical to the formula for the amount of reacted substance in the branching chain reaction. The autocatalyticity factor for two control animals had the following values: phi = 2.5 and 2.0 days$^{\text{minus } 1}$.

For the purpose of describing the dynamics in the growth of hemocytoblasts, in experimental animals, a formula is employed that is analogous to the one that we have obtained for the kinetic curves of branching reactions in the presence of inhibitors (5);

$$G \text{ is approximately equal to } f_2(\text{phi, alpha, } t_1)\, e^{x \text{ phi})t - t_1)}, \qquad (3)$$

where alpha is less than 1, t_1 represents the time of administration of the inhibitor following the transplantation of the leukemia. The product alpha phi = $\text{phi}_{\text{inhibitor}}$ should be of a lesser magnitude than phi for the control animals. This signifies that the rate of development of the transient process, in the presence of an inhibitor, is lower than in its absence. As a matter of fact, for two experimental mice, we obtained the values: $\text{phi}_{\text{inhibitor}}$ = 0.8; 1.0 days$^{\text{minus } 1}$.

On 8-week mice of the afb line, we were able to observe, along with the prolongation of life, also an involution of the leukemic process (recovery), in 9 mice, out of 36, upon their being administered preparations (I) and (II). In Fig. 2 is shown the dynamics of change in the overall number of

leucocytes and in the number of hemocytoblasts in the peripheral blood in two surviving experimental mice upon their being administered preparation (I), which was injected daily over a period of 5 days, in an amount of 3 milligrams for one injection. As may be seen from Fig. 2, the appearance of hemocytoblasts, in the peripheral blood, proceeded against a background of a normal number of leucocytes. One's attention is called to the fact that a complete normalization of the blood has its onset on approximately the 14th day. Analogous observations were made on utilizing preparation (II), which was administered sevenfold in an amount of 4 milligrams for one injection.

A study of the leucocytic formula on blood smears, taken at different periods, indicates that normalization is expressed not only with the disappearance, from the peripheral blood, of immature cellular shapes, but also in the reestablishment of a normal amount of previously drastically declined lymphocytes; the domination of the latter is characteristic for healty mice.

The fact that the surviving mice had actually been ill with leukemia was established, in addition to investigation by methods of customary hematology, also by means of luminescent (scintillation) microscopy of blood smears. The number of hemocytoblasts in the blood, in some mice, reached 17%.

In addition to the survival rate of the experimental animals, we observed, in 28 mice out of an overall number of 51 (lines afb and C_{57}), which had received inhibitors, a prolongation of life by 1.4 - 3.2 times in comparison with the corresponding control. In employing preparation (III) on C_{57} mice, in turn, there were observed instances of blood normalization and that of the bone marrow (Fig. 3). The amount of immature cells, at the height of the process, increased, at the same time, to 21%, i.e., on conversion of 4800 elements for 1 cubic millimeter of blood.

In mice of the C_{57} line, the dimensions of the spleen respresnt a distinct

index of the intensity of the leukemic process. In untreated mice, the spleen proved to be markedly enlarged; the dimensions of the spleen, however, in experimental mice, remained, generally, within the normal range (Fig. 4). ((Illustration on pg. 144 of text)) ((immediately underneath this illustration, in Russian letters)): a; b; v; g; ((below that)): Fig. 4. Spleen in C_{57} mice; a -- control, untreated mouse, on the 8th day following transplantation; b, v -- healthy mice; g -- treated mouse, on the 16th day following transplantation, preparation (II).

((text continued)): As a result of the investigations carried out, we found it possible to obtain, in 8-week mice of the afb line, in 25% of the cases, recovery from leukemia; in 36% of the cases, prolongation of life by 1.5 - 2 times. In mice of the C_{57} line, in 63% of the cases, there was observed a prolongation of life by 1.4 - 3.2 times (in individual cases, with a normalization of the blood and in the cellular composition of the bone marrow).

Great interest is found in the results of experiments in which we attempted to produce leukemia, for a second time, in mice that had receovered. All the recovered mice proved to be resistant to secondary inoculations of leukemia. This resistance has already been maintained for a prolonged time (6 months). Moreover, the following generation of animals, obtained from a cross-(inter) breeding of these mice, likewise proved to be resistant relative to transplantable leukemia.

The results thus obtained attest to the necessity of setting up broad investigations of the antitumoral activity of strong nontoxic inhibitors from chain oxidative processes (in the first place, from a type of food antioxidants). If the general consensus, which made it possible to begin this work, turns out to be correct, then it may be expected that the class of chemotherapeutic drugs, such as we have proposed, will prove to be effective for a very broad spectrum of malignant tumors.

The authors wish to express their appreciation to Z. D. Shostakovich and to I. I. Pelevina (Laboratory of Anisotropic Structures of the AN SSSR (Academy of Sciences of the U.S.S.R.) for their assistance in carrying out the experiments, and also, to E. E. Pogosyants (Institute of Experimental Cancer Pathology and Therapy of the AMN SSSR (Academy of Medical Sciences of the U.S.S.R.) for making available the leukemic strains.

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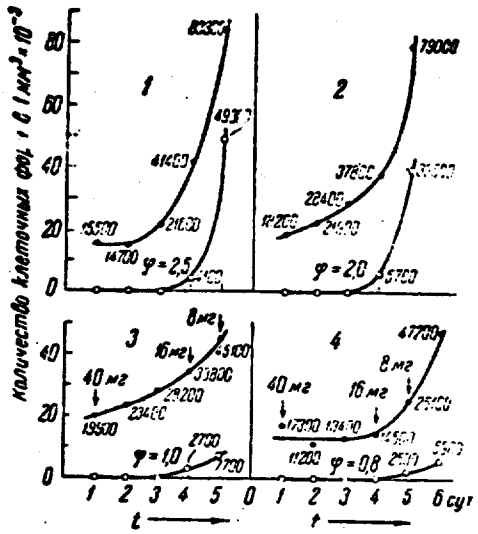

Figure 1

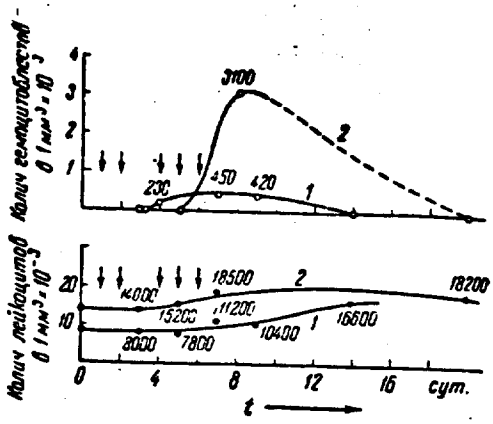

Figure 2

Figure 3

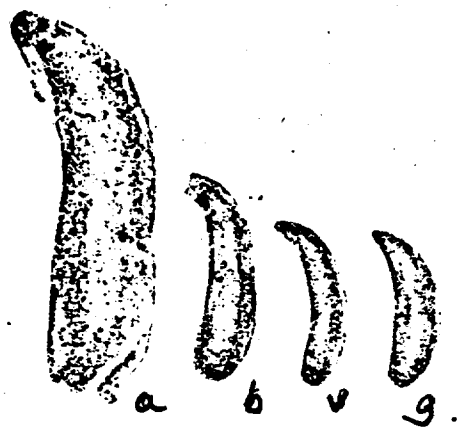

Figure 4

...лительном озвучивании в
...чество кислорода за счет
...ствл...ого в кавитацион-
...в ра...воре водорода, как
...еса озвучиваемого протеи-
...розрачным, а озвученный
...ся на поверхности исполь-
...ем молекулярный вес сы-
...чивался приблизительно
...взаимодействии осколков
...Такому взаимодействию,

...ода, по-видимому, осуще-

Таблица 3

...присутствии различных газов.
...щадь белковой пленки при $F = 0$

...Газ, кото-...рым насы-...щен озвуч. раствор	Т-ра, °С	Мол. вес	Измен. веса, %
H_2	22	122570	+65
N_2	23	73400	0
N_2	22	76000	0
N_2	24	59050	—24

...озможности изучения кор-
...х те...

Поступило
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...УРА

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Доклады Академии наук СССР
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БИОХИМИЯ

Н. М. ЭМАНУЭЛЬ и Л. П. ЛИПЧИНА

ЛЕЙКОЗ У МЫШЕЙ И ОСОБЕННОСТИ ЕГО РАЗВИТИЯ ПРИ ВОЗДЕЙСТВИИ ИНГИБИТОРОВ ЦЕПНЫХ ОКИСЛИТЕЛЬНЫХ ПРОЦЕССОВ

(Представлено академиком Н. Н. Семеновым 6 V 1958)

Прогрессивное развитие злокачественного опухолевого процесса находит много аналогий в протекании нестационарных химических процессов, например, цепных разветвленных реакций, открытых Н. Н. Семеновым ([1]). Не исключена возможность, что в основе наблюдаемых аналогий лежит сходство природы активных промежуточных веществ, ответственных за самоускоренный характер развития процессов in vivo и in vitro. Активными центрами цепных реакций являются свободные радикалы. В медленно развивающихся (не взрывных) цепных разветвленных реакциях образуются кроме того промежуточные вещества, изредка распадающиеся с образованием свободных радикалов, в результате чего возникают вырожденные (запаздывающие) разветвления и процесс медленно самоускоряется во времени.

Имеются вполне определенные указания, что в механизмах некоторых биохимических процессов играют роль свободные радикалы и цепные реакции ([2]). Особенно существенно, что с этой точки зрения рассматриваются реакции биологического (ферментативного) окисления, которое как раз и дает необходимую энергию для роста злокачественных новообразований.

Нельзя также не обратить внимания на то, что во многих случаях механизм канцерогенеза можно представить себе, исходя из возможности появления в биосубстрате свободных радикалов и стимулирования ими радикальных (цепных) реакций ([3]).

Ряд фактов может служить подтверждением этого взгляда. Воздействие проникающих излучений на живой организм, сводящееся в конечном счете к образованию свободных радикалов и атомов, способно вызвать лучевой рак и лейкоз. Канцерогенные (полициклические) углеводороды сильно стимулируют различные биологические эффекты, идущие под действием света (фотодинамический эффект). Сенсибилизирующее действие канцерогенов рассматривается как результат перехода их при поглощении кванта света в триплетное (бирадикальное) состояние, что находится в соответствии с общей теорией действия фотосенсибилизаторов А. Н. Теренина ([4]). Принято считать, что при подходящих условиях образование свободных радикалов может иметь место и в отсутствие света (при введении канцерогенов) в результате взаимодействия канцерогенного углеводорода с подходящим субстратом. Зависимость частоты заболевания раком кожи от интенсивности воздействия даже солнечного излучения позволяет говорить о возникновении в эпидермисе фотохимических радикальных реакций, стимулирующих патологический процесс.

Представление о свободно радикальном механизме канцерогенеза и развития патологического процесса, а также аналогия с цепными вырожденно-разветвленными реакциями естественно приводят к выводу о целесообразности попыток воздействия на рост злокачественных новообразований путем введения в организм веществ, легко взаимодействующих со свободными радикалами (обрывающих цепи).

Основным источником энергии для синтеза больших масс белка является биологическое окисление. Поэтому для числа размножающихся, растущих, а в ряде случаев также дифференцирующихся клеток G можно записать

соотношение

$$G \sim \Delta F \sim \int w_{окисл} dt, \qquad (1)$$

где $w_{окисл}$ — скорость биологического окисления, стимулируемого (по предположению) включением свободно радикальных реакций.

В качестве веществ, содержащих реакционноспособные группы, реагирующие со свободными радикалами, мы решили испытать сильные нетоксичные

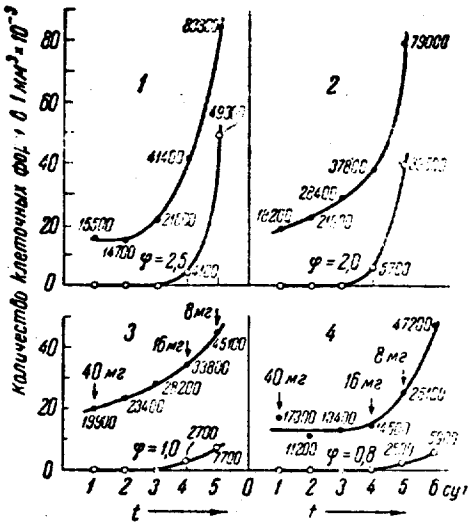


Рис. 1. Динамика нарастания общего количества лейкоцитов (черные точки) и незрелых клеток (кружки) в 1 мм³ крови у 6-недельных мышей линии afb. Контроль (*1*, *2*) и опыт при воздействии препарата (II) (*3*, *4*). Стрелками показаны моменты введения препарата

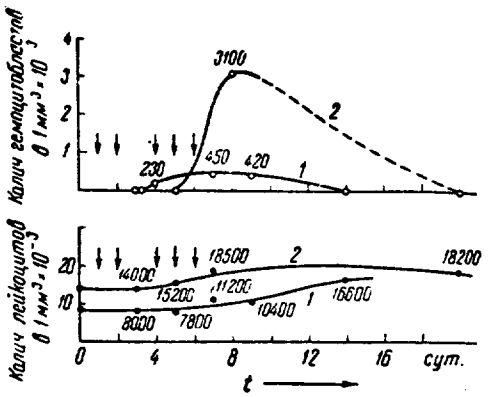


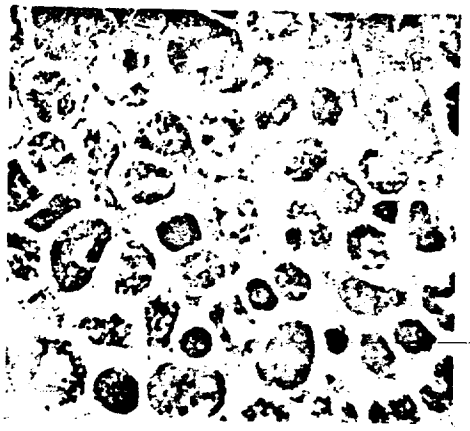
Рис. 2. Динамика изменения содержания лейкоцитов (черные точки) и незрелых клеток (кружки) в 1 мм³ крови у двух выздоровевших 8-недельных мышей линии afb в результате воздействия препарата (I). Стрелками показаны моменты введения препарата в количестве 3 мг в инъекцию

ингибиторы цепных окислительных реакций (пищевые антиокислители): (I) бутилоксианизол (смесь двух изомеров: 2- и 3-трет. бутил-4-оксианизолов); (II) ионол (2,6-ди-трет. бутил-4-метилфенол); (III) пропилгаллат (нормальный пропиловый эфир галловой кислоты) и др. Наличие в этих соединениях сильных ориентантов первого рода в орто- и параположениях и их экранирующее действие обусловливают высокую реакционную способность водорода гидроксильной группы. Для изучения воздействия ингибиторов цепных окислительных процессов на течение и исход опухолевой болезни мы использовали перевиваемые остротекущие лейкозы у высоколейкозных мышей линии afb, штамм ЛIV и у практически нелейкозных черных мышей линии C_{57}, штамм ЛА, полученный при перевивке лейкоза, вызванного у мышей линии C_{57} рентгеновскими лучами.

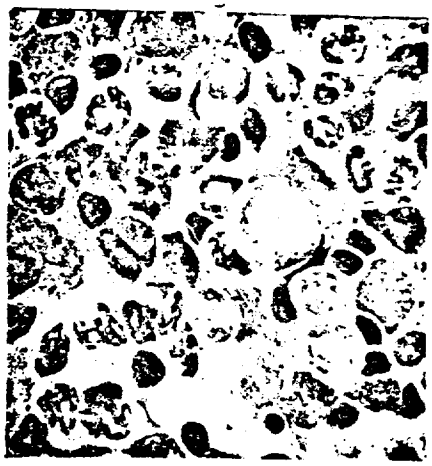
Перевиваемый лейкоз — удобная экспериментальная опухолевая модель: гематологические исследования позволяют следить за течением опухолевого процесса по количественным и качественным сдвигам клеточных форм.

Эксперименты велись на мышах 6—8-недельного возраста весом от 15 до 23 г. Перевивка производилась внутрибрюшинно, по обычно принятой методике, которая обеспечивает 100%-ю перевиваемость лейкоза и 100%-ю гибель мышей. 6-недельные мыши (15—17 г) погибали на 5—6 день после перевивки, 8-недельные (18—23 г) — на 8—10 день. Всего под опытом было 198 линейных мышей. В терапевтических опытах 8-недельных мышей линии afb было 70 (36 опытных, 34 контрольных), мышей линии C_{57}—48 (24 опытных, 24 контрольных), остальные мыши (6—8-недельные) использовались для подбора дозировок и изучения биологического действия препаратов.

а



б



в

Рис. 3. Картина костного мозга у мышей линии C_{57}. *а* — контрольная нелеченная мышь; *б* — мышь, получавшая препарат (III); *в* — здоровая мышь. Об. имм. 60, ок. 10 ×. Микроскоп Цейсс

Введение препаратов начиналось со 2-го дня после перевивки. Препараты (I) и (II) подкожно инъецировались 3—10 раз, препарат (III) вводился внутрибрюшинно в течение 5 дней. Препараты (I), (II) растворялись в абрикосовом масле, препарат (III)—в физиологическом растворе. Препарат (I) вводился из расчета 150 мг/кг в каждую инъекцию; (II)— 200 мг/кг; (III)—37,5 мг/кг. Контрольным мышам инъецировались соответствующие количества растворителя. В некоторых опытах разовые дозировки препаратов (I) и (II) повышались в 2—10 раз. При больших дозировках у мышей 15—17 г отмечались падение в весе и явления диспепсии; более крупные мыши высокие дозы препарата переносили хорошо. Химиотерапевтический индекс (ДЛ : ДПО) для препарата (I) равняется 20, а для препарата (II) 30.

У здоровых мышей как при разовом введении 30 мг препарата (I) и 80 мг препарата (II), так и при хроническом введении в течение 2 мес. 3—8 мг препаратов (I) и (II) падения в весе и явлений диспепсии не наблюдалось, кровь и костный мозг оставались в пределах нормы, патологических изменений со стороны внутренних органов не отмечалось.

На рис. 1 показан пример нарастания общего числа лейкоцитов и отдельно незрелых клеток (гемоцитобластов и лимфобластов) в периферической крови при перевиваемом лейкозе у мышей линии aIb без введения препарата (контроль) и при трехкратных инъекциях препарата (II) из расчета 8—40 мг на мышь за одну инъекцию. В этом опыте были использованы 6-недельные мыши (15—16 г), у которых, как уже указывалось, лейкоз развивается исключительно остро. Поэтому полученные данные следует рассматривать только с точки зрения оценки влияния препарата на динамику гематологических изменений. Отчетливо видно торможение процесса нарастания как общего числа лейкоцитов, так и незрелых клеток. Рост числа незрелых клеток (гемоцитобластов) описывается формулой

$$G \approx f_1(\varphi)\, e^{\varphi t}, \qquad (2)$$

математически тождественной с формулой для количества прореагировавшего вещества в цепной разветвленной реакции. Фактор автокаталитичности для двух контрольных животных имел следующие значения: $\varphi = 2{,}5$ и $2{,}0$ сутки$^{-1}$.

Для описания динамики нарастания гемоцитобластов у опытных животных применима формула, аналогичная полученной нами для кинетических кривых цепных разветвленных реакций в присутствии ингибиторов ([5]):

$$G \approx f_2(\varphi, \alpha, t_1)\, e^{\alpha\varphi(t-t_1)}, \qquad (3$$

где $\alpha < 1$, t_1 — время введения ингибитора после перевивки лейкоза. Произведение $\alpha\varphi = \varphi_{\text{ингиб}}$ должно быть меньшей величиной, чем φ для контрольных животных. Это означает, что скорость развития нестационарного процесса в присутствии ингибитора меньше, нежели в его отсутствие. Действительно, для двух опытных мышей мы получили значения: $\varphi_{\text{ингиб}} = 0{,}8$; $1{,}0$ сутки$^{-1}$.

На 8-недельных мышах линии aIb нам удалось наблюдать наряду с продлением жизни также обратное развитие лейкозного процесса (выздоровление) у 9 мышей из 36 при введении им препаратов (I) и (II). На рис. 2 показана динамика изменения общего количества лейкоцитов и числа гемоцитобластов в периферической крови у двух выживших опытных мышей при введении им препарата (I), который инъецировался ежесуточно в течение 5 дней в количестве 3 мг за одну инъекцию. Как видно из рис. 2, появление гемоцитобластов в периферической крови шло на фоне нормального количества лейкоцитов. Обращает на себя внимание полная нормализация крови, наступающая примерно на 14 сутки. Аналогичные наблюдения были сделаны при использовании препарата (II), который вводился семикратно в количестве 4 мг за одну инъекцию.

Изучение лейкоцитарной формулы на мазках крови, взятых в различные сроки, показывает, что нормализация выражается не только в исчез-

новении из периферической крови незрелых клеточных форм, но и в восстановлении нормального количества ранее резко упавших лимфоцитов, доминирование которых характерно для здоровых мышей.

Факт, что выжившие мыши действительно болели лейкозом, устанавливался помимо исследования методами обычной гематологии также с помощью люминесцентной микроскопии мазков крови. Количество гемоцитобластов в крови у некоторых мышей достигало 17%.

Кроме выживаемости подопытных животных мы наблюдали у 28 мышей из общего числа 51 (линий afb и C_{57}), получавших ингибиторы, продление жизни в 1,4 — 3,2 раза по сравнению с соответствующим контролем. При применении препарата (III) на мышах C_{57}, в свою очередь, наблюдались случаи нормализации крови и костного мозга (рис. 3). Количество незрелых клеток в разгар процесса поднималось при этом до 21%, т. е. при пересчете на 1 мм³ крови 4800 элементов.

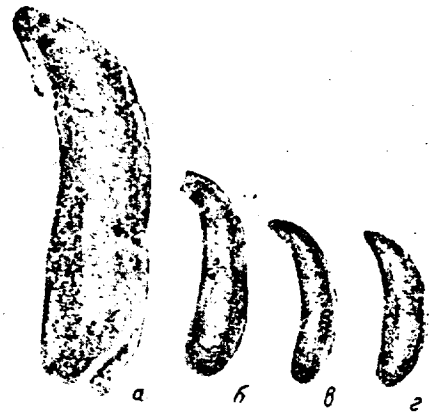


Рис. 4. Селезенка у мышей C_{57}. *а* — контрольная нелеченная мышь, 8-й день после перевивки; *б*, *в* — здоровые мыши; *г* — леченная мышь, 16-й день после перевивки, препарат (II)

У мышей линии C_{57} размеры селезенки являются отчетливым показателем интенсивности лейкозного процесса. У нелеченных мышей селезенка оказывалась резко увеличенной, размеры же селезенки у опытных мышей оставались обычно в пределах нормы (рис. 4).

В результате проведенных исследований нам удалось получить на 8-недельных мышах линии afb в 25% случаев выздоровление от лейкоза, в 36% случаев продление жизни в 1,5—2 раза. На мышах линии C_{57} в 63% случаев наблюдалось продление жизни в 1,4—3,2 раза (в отдельных случаях с нормализацией крови и клеточного состава костного мозга).

Большой интерес представляют результаты опытов, в которых мы пытались повторно вызвать лейкоз у вылеченных мышей. Все вылеченные мыши оказались резистентными к повторным прививкам лейкоза. Эта устойчивость сохраняется уже длительное время (6 месяцев). Более того, следующее поколение животных, полученное от скрещивания этих мышей, также оказалось резистентным по отношению к перевиваемому лейкозу.

Полученные результаты свидетельствуют о необходимости развернуть широкие исследования противоопухолевой активности сильных нетоксичных ингибиторов цепных окислительных процессов (в первую очередь типа пищевых антиокислителей). Если общая концепция, которая позволила начать эту работу, окажется правильной, то можно ожидать, что предложенный нами класс химиотерапевтических средств окажется эффективным для весьма широкого спектра злокачественных опухолей.

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Институт химической физики
Академии наук СССР

Лаборатория анизотропных структур
Академии наук СССР

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ЦИТИРОВАННАЯ ЛИТЕРАТУРА

[1] Н. Н. Семенов, Цепные реакции, Л., 1934. [2] У. Уотерс, Химия свободных радикалов, ИЛ, 1948. [3] Успехи в изучении рака, 2, ИЛ, 1956; W. Gordy, Simposium on Information Theory in Biology Gatlinburg, 1956. [4] А. Н. Теренин, Фотохимия красителей, Изд. АН СССР, 1947. [5] Н. М. Эмануэль, ДАН, 95, 603 (1954).

d_4^{25} (2. n_D^{20} 1.3970.
eroux, Lucas, *loc. cit.*
agents, flavors, perfumes,
industrial cleaners, paint
tural resins, linseed and
n-Butyl Alcohol.

-yl-2-propanol; trimethyl
al formula, mol wt, etc.:
acetyl chloride and di-
1 (1867). Manuf: by
Kreps, Nachod, U.S.
Refining Co.), Serniuk,
(1950 to Standard Oil
tert-butyl hydroperoxide,
to Hercules Powder Co.),
58 to Shell Development
or pressure: Biddiscombe

Flash pt 25.7°. At 99.69
0.78581, d_4^{25} 0.78086, d_4^{25}
n_D^{25} 1.38231: Dreisbach,
(1949). Soluble in water.
$_{50}$ orally in rats: 3.5 g/kg.
mfg flotation agents,
paint removers. *Human*

-aminobutane. CH_3CH_2-
$C_4H_{11}N$; C 65.69%, H
induction of butyraldoxime:
2, 319 (1943). Usually
of ammonia with butyl
2,609,394 (1952 to I.C.I.,
2,782,237 (1957 to British
U.S. pat. 3,022,349 (1962
rley, Speranza, U.S. pat.
mical Co., Inc.). Manuf
in the presence of Raney
518,6[illegible] (1950 to Eastman

78°. −50°. d_4^{25} 0.7327.
er, alcohol, ether. LD_{50}

ceuticals, dyestuffs, rubber
secticides, synthetic tanning
irritating to skin, mucous

tane. $CH_3CH_2CH(NH_2)$-
wt etc.: *see n*-butylamine.
hyl ketoxime: Lycan *et al.*,
3). Manuf: Taylor *et al.*,
Ltd.); Thurston, U.S. pat.
mid Co.). Resolution of
30); Bruck *et al.*, *J. Chem.*

−104°. d_4^{20} 0.724. n_D^{20} 1.394.

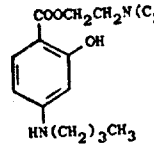
d_4^{15} 0.7308. n_4^{15} 1.3963.

0.728. $[\alpha]_D^{19}$ −7.64°. *Human*

sobutane; 2-amino-2-methyl-
rical formula, mol wt, etc.:
mpbell *et al.*, *Org. Syn.* coll.
am *et al.*, U.S. pat. 2,501,509
Kibler, U.S. pat. 2,773,097
per Co.).
65°. d_4^{20} 0.6951, d_4^{25} 0.6867.

amine.

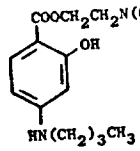
2-Diethylaminoethyl Ester.
acid 2-diethylaminoethyl
tylaminosalicylate; Broncho-
$C_{17}H_{28}N_2O_3$; mol wt 308.41.
O 15.56%. Prepn: Grimme,
Brit. pat. 760,003 (1956 to



Hydrochloride, $C_{17}H_{28}N_2O_3.HCl$, crystals from isopropanol or prisms from water, mp 138°. Soluble in water. Aq solns should be prepd just prior to using.

MED USE: Surface anesthetic, especially vaginal, rectal. *Dose:* Topically 75 mg as suppository.

p-Butylaminosalicylic Acid 2-Dimethylaminoethyl Ester. 4-Butylamino-2-hydroxybenzoic acid 2-dimethylaminoethyl ester; 2-dimethylaminoethyl *p*-butylaminosalicylate; hydroxamethocaine; Rhenocain. $C_{15}H_{24}N_2O_3$; mol wt 280.36. C 64.26%, H 8.63%, N 9.99%, O 17.12%. Prepn: Brit. pats. 736,960 (1955) and 760,003 (1956 to Rheinpreussen AG); Grimme, Schmitz, *Ber.* 84, 734 (1951).

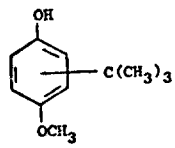


Hydrochloride, $C_{15}H_{24}N_2O_3.HCl$, crystals from water, mp 157°. Solubility in water at 20°: about 4%.

Hemihydrate, $C_{15}H_{24}N_2O_3.\frac{1}{2}H_2O$, prisms from ligroin, mp 48°.

MED USE: Local anesthetic.

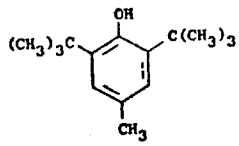
Butylated Hydroxyanisole. BHA. A mixture of *2-tert-butyl-4-methoxyphenol* (also called 3-*tert*-butyl-4-hydroxyanisole) and *3-tert-butyl-4-methoxyphenol* (also called 2-*tert*-butyl-4-hydroxyanisole); Embanox. $C_{11}H_{16}O_2$; mol wt 180.24. C 73.30%, H 8.95%, O 17.75%. Prepn from *p*-methoxyphenol and isobutene: Rosenwald, U.S. pats. 2,459,540 and 2,470,902 (1949 to Universal Oil Products Co.).



Insoluble in water. Soluble in petr ether (Skellysolve H), in 72% alcohol (or higher), in propylene glycol. Soluble in fats and oils to some extent (<0.5%). Exhibits antioxidant properties and synergism with acids, hydroquinone, methionine, lecithin, thiodipropionic acid, etc. Determination using 2,6-dichloroquinonechlorimide: Mahon, Chapman, *Anal. Chem.* 23, 1116, 1120 (1951).

USE: Antioxidant, esp in foods. The American Meat Institute Foundation has proposed an antioxidant mixture known as AMIF-72 which contains 20% of butylated hydroxyanisole, 6% of propyl gallate, and 4% of citric acid in propylene glycol. This formula is widely used in commercial antioxidant prepns, *e.g.*, *Sustane*, *Tenox*.

Butylated Hydroxytoluene. BHT; DBPC; *2,6-di-tert-butyl-p-cresol;* 2,6-di-*tert*-butyl-4-methylphenol; Ionol; Dalpac; Impruvol; Vianol. $C_{15}H_{24}O$; mol wt 220.34. C 81.76%, H 10.98%, O 7.26%. Prepd from *p*-cresol and isobutylene: Stillson, U.S. pat. 2,428,745 (1947 to Gulf). McConnell, Davis, U.S. pat. 3,082,258 (1963 to Eastman Kodak Co.).



Crystals, mp 70°. d_4^{20} 1.048. bp 265°. Flash pt open cup: 260°F (127°C). Insoluble in water. Freely sol in toluene, sol in methanol, ethanol, isopropanol, ethyl methyl ketone, acetone, Cellosolve, petr ether, benzene, most other hydrocarbon solvents. Solubility in liquid petrolatum (white oil): 0.5% w/w. More sol in food oils and fats than butylated hydroxyanisole. Good soly in linseed oil. LD_{50} in rats: >1500 mg/kg.

USE: Antioxidant for food, animal feed, petr products, synth rubbers, plastics, animal and vegetable oils, soaps. Antiskinning agent in paints and inks. *Human Toxicity:* May cause sensitization type of dermatitis. Practically no systemic toxicity.

***n*-Butylbenzene.** *1-Phenylbutane.* $C_6H_5CH_2CH_2$-CH_2CH_3; mol wt 134.21. $C_{10}H_{14}$; C 89.49%, H 10.51%. Prepd with almost quantitative yield from benzyl chloride or bromide, propyl bromide and sodium without additional solvents: Radziszewski, *Ber.* 9, 261 (1876). Similarly from bromobenzene, butyl bromide and sodium in benzene: Balbiano, *Ber.* 10, 296 (1877); Read, Foster, *J. Am. Chem. Soc.* 48, 1606 (1926).

Liquid. mp −88.5°. d_4^{20} 0.8604. bp_{760} 183.1°; bp_{400} 159.2°; bp_{200} 136.9°; bp_{100} 116.2°; bp_{60} 102.6°; bp_{40} 92.4°; bp_{20} 76.3°; bp_{10} 62.0°; bp_5 48.8°; $bp_{1.0}$ 22.7°. n_D^{20} 1.49040. Insoluble in water; miscible with alcohol, ether, benzene.

***sec*-Butylbenzene.** *2-Phenylbutane.* $C_6H_5CH(CH_3)$-CH_2CH_3; mol wt 134.21. $C_{10}H_{14}$; C 89.49%, H 10.51%. Prepd from benzene and *n*-butyl chloride in presence of $AlCl_3$: Schramm, *Monatsh.* 9, 621 (1888); by the action of sodium on γ-chloro-*sec*-butylbenzene: Braun, Grabowski, Kirschbaum, *Ber.* 46, 1277 (1913); with other products by heating *n*- or *sec*-butyl alcohol with 80% H_2SO_4: Meyer, Bernhauer, *Monatsh.* 53, 727 (1929).

Liquid. mp −82.7°. d_4^{20} 0.8608. bp_{760} 173.5°; bp_{400} 150.3°; bp_{200} 128.8°; bp_{100} 109.5°; bp_{60} 96.0°; bp_{40} 86.2°; bp_{20} 70.6°; bp_{10} 57.0°; bp_5 44.2°; $bp_{1.0}$ 18.6°. n_D^{20} 1.48980. Insoluble in water; miscible with alcohol, ether, benzene.

d-Form: $[\alpha]_D^{20}$ +1.36°.

l-Form: $[\alpha]_D^{23}$ −2.30°.

USE: Solvent; in organic syntheses.

***tert*-Butylbenzene.** *2-Methyl-2-phenylpropane;* trimethylphenylmethane; pseudobutylbenzene. $C_6H_5C(CH_3)_3$; mol wt 134.21. $C_{10}H_{14}$; C 89.49%, H 10.51%. Prepd from benzene, isobutyl chloride and $AlCl_3$: Konowalow, *Bull. Soc. Chim.* [3] 16, 865 (1896); Shoesmith, Mackie, *J. Chem. Soc.* 1928, 2336; from isobutyl alcohol and benzene by treatment with fuming sulfuric acid: Meyer, Bernhauer, *Monatsh.* 53, 727 (1929). *See also* Groose, Ipatieff, *J. Am. Chem. Soc.* 57, 2416 (1935); Ipatieff, Pines, *ibid.* 58, 1056 (1936).

Liquid. mp −58.1°. d_4^{20} 0.8669. bp_{760} 168.5°; bp_{400} 145.8°; bp_{200} 123.7°; bp_{100} 103.8°; bp_{60} 90.6°; bp_{40} 80.8°; bp_{20} 65.6°; bp_{10} 51.7°; bp_5 39.0°; $bp_{1.0}$ 13.0°. n_D^{20} 1.49235. Insoluble in water; miscible with alcohol, ether, benzene.

***n*-Butyl Benzoate.** *Benzoic acid butyl ester.* C_6H_5COO-$(CH_2)_3CH_3$; mol wt 178.22. $C_{11}H_{14}O_2$; C 74.13%, H 7.92%, O 17.95%. Prepn: Newman, Fones, *J. Am. Chem. Soc.* 69, 1046 (1947); Justoni, Brit. pat. 719,891 (1954 to Vismara).

Thick, oily liquid. d 1.00. mp −22°. bp 250°. Practically insol in water; sol in alcohol or ether.

Human Toxicity: Animal expts show fairly low toxicity.

***n*-Butyl Bromide.** *1-Bromobutane.* $CH_3CH_2CH_2CH_2Br$; mol wt 137.03. C_4H_9Br; C 35.06%, H 6.62%, Br 58.32%. Prepd from *n*-butyl alcohol and a hydrobromic-sulfuric acid mixture: Kamm, Marvel, *Org. Syn.* vol. 1, p 5 (1921). Skau, McCullough, *J. Am. Chem. Soc.* 57, 2440 (1935).

Colorless liquid. d_4^{25} 1.2686. bp_{760} 101.3° (mp −112°). n_D^{20} 1.4398. Insoluble in water; sol in alcohol, ether.

***sec*-Butyl Bromide.** *2-Bromobutane;* methylethylbromomethane. $CH_3CH_2CHBrCH_3$; mol wt 137.03. C_4H_9Br; C 35.06%, H 6.62%, Br 58.32%. Prepn: Levene, Marker, *J. Biol. Chem.* 91, 405 (1931); Kenyon, Phillips, Pittman, *J. Chem. Soc.* 1935, 1080; Skau, McCullough, *J. Am. Chem. Soc.* 57, 2440 (1935).

dl-Form: Colorless liquid, pleasant odor. d_4^{25} 1.2530. bp 91.2° (mp −112°). n_D^{25} 1.4344. Insoluble in water. Freely sol in alcohol, ether.

d-Form: d_4^{25} 1.251. bp 91°. $[\alpha]_D^{25}$ +10.81°.

ed in the general method,
70 per cent alcohol, and

able method (see page 4).
the general method, page

as directed for organic
rsenic Test, page 720.
mg. sample as directed in
e 763, using 20 mcg. of lead

directed for organic com-
Limit Test, page 772, using

ght, preferably glass, tin-
e protected from light.
t.

BLE OIL

ition product of vegetable
viscous, oily liquid having
t is insoluble in water, but
in hexane, and in fixed oils.

ram of anhydrous sodium
mixture with an additional
mixture by gentle tapping,
for 10 minutes. Cool the
ue in 20 ml. of hot water,
c acid T.S. until efferves-
e T.S. A curdy, yellowish
but soluble in an excess of

ed by the vendor.

ts per million (0.0003 per

Free fatty acids (as oleic). Not more than 1 per cent.
Heavy metals (as Pb). Not more than 10 parts per million (0.001 per cent).
Iodine value. Not more than 1.

TESTS

Specific gravity. Determine as directed in the general procedure, page 761, except make the determination at 20°/20°.

Arsenic. A *Sample Solution* prepared as directed for organic compounds meets the requirements of the *Arsenic Test*, page 720.

Free bromine. Dissolve 1 gram in 20 ml. of acetone, add 1 gram of sodium iodide, and allow to stand in a stoppered flask in the dark for 30 minutes, with occasional shaking. Add 25 ml. of water and 1 ml. of starch T.S. No blue color is produced.

Free fatty acids. Determine as directed in the general procedure, page 749, using 28.2 as the equivalence factor (*e*) in the calculation for oleic acid.

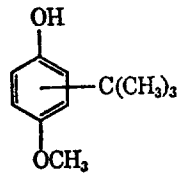
Heavy metals. Prepare and test a 2-gram sample as directed in *Method II* under the *Heavy Metals Test*, page 763, using 20 mcg. of lead ion (Pb) in the control (*Solution A*).

Iodine value. Determine by the *Wijs Method*, page 752.

Packaging and storage. Store in well-closed containers.
Functional use in foods. Flavoring adjunct; beverage stabilizer.

BUTYLATED HYDROXYANISOLE

BHA



$C_{11}H_{16}O_2$ Mol. wt. 180.25

DESCRIPTION

Butylated hydroxyanisole is predominately 3-*tert*-butyl-4-hydroxyanisole (3-BHA), with varying amounts of 2-*tert*-butyl-4-hydroxyanisole (2-BHA). It occurs as a white or slightly yellow, waxy solid having a faint characteristic odor. It is insoluble in water, but is freely soluble in alcohol and in propylene glycol. It melts between 48° and 63°.

IDENTIFICATION

To 5 ml. of a 1 in 10,000 solution of the sample in 72 per cent alcohol add 2 ml. of sodium borate T.S. and 1 ml. of a 1 in 10,000 solution of 2,6-dichloroquinonechlorimide in absolute alcohol, and mix. A blue color develops.

SPECIFICATIONS

Assay. Not less than 98.5 per cent of $C_{11}H_{16}O_2$.

Limits of Impurities

Arsenic (as As). Not more than 3 parts per million (0.0003 per cent).

Heavy metals (as Pb). Not more than 10 parts per million (0.001 per cent).

Residue on ignition. Not more than 0.01 per cent.

TESTS

Assay

Standard Reference Curve. Weigh accurately 900.0 mg., 950.0 mg., and 1.0000 gram of F.C.C. 3-*tert*-Butyl-4-hydroxyanisole Reference Standard, representing 90, 95, and 100 per cent of $C_{11}H_{16}O_2$, respectively, into separate 10-ml. volumetric flasks. Dissolve each standard in carbon disulfide, dilute to volume with carbon disulfide, and mix. Measure the infrared absorption spectrum of each solution from 10.5 μ to 12.5 μ with a suitable double beam infrared spectrophotometer, using 0.15 mm. sample cells, a 1.3 cm. rock salt plate in the reference beam, 2 $\times$ slits, and normal scanning speed. Draw a background line on the spectrogram from 11.2 μ to 12.0 μ, and determine the net absorbance of each solution at 11.42 μ by subtracting the background absorbance at this wavelength from the total absorption of each solution. Plot the calculated net absorbances against the per cent of $C_{11}H_{16}O_2$ in each solution.

Assay Preparation. Transfer 1.0000 gram of the sample, accurately weighed, into a 10-ml. volumetric flask, dissolve it in carbon disulfide, dilute to volume with carbon disulfide, and mix. Measure the infrared absorption spectrum of this solution using the same conditions described above, determine the net absorbance of the sample at 11.42 μ, and obtain the apparent percentage of $C_{11}H_{16}O_2$ by means of the *Standard Reference Curve.*

Calculation. Calculate the true, total percentage of $C_{11}H_{16}O_2$ in the sample of butylated hydroxyanisole taken by the formula [(Apparent % BHA) + 0.16(100 − % 3-BHA)], in which *Apparent % BHA* is the percentage found by the procedure described under *Assay Preparation,* 0.16 is a factor to correct for the decreased absorbance of the 2-isomer (which absorbs only 84 per cent as strongly as does the 3-isomer at 11.42 μ), and *% 3-BHA* is the percentage of 3-*tert*-butyl-4-hydroxyanisole in the sample obtained by the procedure described below.

nple in 72 per cent alcohol
f a 1 in 10,000 solution of
ıcohol, and mix. A blue

$_{16}O_2$.

s per million (0.0003 per

0 parts per million (0.001

.01 per cent.

ely 900.0 mg., 950.0 mg.,
ydroxyanisole Reference
cent of $C_{11}H_{16}O_2$, respec-
.. Dissolve each standard
arbon disulfide, and mix.
f each solution from 10.5
rared spectrophotometer,
salt plate in the reference
d. w a background
, determine the net
tracting the background
absorption of each solu-
against the per cent of

of the sample, accurately
ive it in carbon disulfide,
ix. Measure the infrared
the same conditions de-
e of the sample at 11.42
$_{11}H_{16}O_2$ by means of the

centage of $C_{11}H_{16}O_2$ in the
the formula [(Apparent
Apparent % BHA is the
under *Assay Preparation*,
osorbance of the 2-isomer
as does the 3-isomer at
of 3-*tert*-butyl-4-hydroxy-
ure described below.

% 3-BHA. Accurately weigh 1.0000 gram of the sample, previously melted in a water bath and thoroughly mixed, transfer it into a 10-ml. volumetric flask, dilute to volume with carbon disulfide, and mix. Measure the infrared absorption spectrum of this solution from 10 μ to 12 μ, using a 0.4 mm. sample cell and a 1.3 cm. rock salt plate in the reference beam. Subtract the background absorbance at 10.40 μ from the absorbance at 10.75 μ and at 10.95 μ. Using these net absorbance values, calculate the absorbance ratio $A_{10.75}/A_{10.95}$ by dividing the net absorbance found at 10.75 μ by that found at 10.95 μ. (The exact position of these absorption bands may vary, depending upon the instrument. If a recording instrument is used, the position of maximum absorbance on the spectrogram should be taken. With a non-recording instrument, the exact wavelength and slit settings should be determined.) Determine the percentage of 3-*tert*-butyl-4-hydroxyanisole in the sample taken by means of a calibration curve obtained as follows: Prepare three 10.0-ml. solutions in carbon disulfide containing, respectively, the following quantities of the specified F.C.C. Reference Standards: (1) 1.0000 gram of 3-*tert*-butyl-4-hydroxyanisole, (2) 900.0 mg. of 3-*tert*-butyl-4-hydroxyanisole and 100.0 mg. of 2-*tert*-butyl-4-hydroxyanisole, and (3) 800.0 mg. of 3-*tert*-butyl-4-hydroxyanisole and 200.0 mg. of 2-*tert*-butyl-4-hydroxyanisole. Measure the infrared absorption spectrum of each solution under the same conditions employed for the sample, and plot the calculated absorbance ratios against the corresponding percentages of 3-*tert*-butyl-4-hydroxyanisole.

Arsenic. A *Sample Solution* prepared as directed for organic compounds meets the requirements of the *Arsenic Test*, page 720.

Heavy metals. Prepare and test a 2-gram sample as directed in *Method II* under the *Heavy Metals Test*, page 763, using 20 mcg. of lead ion (Pb) in the control (*Solution A*).

Residue on ignition. Ignite 10 grams as directed in the general method page 786.

Packaging and storage. Store in well-closed containers.

Functional use in foods. Antioxidant.

ary canal. Hence it is unlikely
oxidant that was eliminated in
ince rats have no gall bladder,
d a small degree of absorption
ecially if entero-hepatic circu-
^{14}C in the 24-hr bile of rats
dosed orally with [^{14}C] Ionox
onox 330 is not absorbed from
ile was not available to assist
eriment with rats with biliary
d from pigs with biliary fistu-
stomach tube after treatment
330. In comparison with the
fistulae, the pigs with biliary
ost-operatively and maintained
ghout the experiment. The ab-
igs confirms that [^{14}C] Ionox
rointestinal tract.
ity of Ionox 312 and Ionox 330,
in male and female rats that
r after the ingestion of [^{14}C]
ed in the urine and ex-
absence of radioactivity in the
iscera after removal of the
otal elimination could have oc-
ng [^{14}C] Ionox 312 was still
ongly suggests the absence of
e of ^{14}C in the 24-hr bile of
previously been dosed orally
t [^{14}C] Ionox 312 is not ab-
ract.
of Ionox 330 and Ionox 312
ecules at the pH of gastric and
ompounds were not absorbed
x 312 were precipitated from
d is absorbed. Foreign organic
eir properties are similar to
abolites, for which there are
their size, shape, and colloidal
2 are unlikely to be absorbed
e (see Wright *et al.*, 1965a).
ects of alimentary absorption.

Ionox 330 is not metabolized by animals, because of its non-absorption and because of its stability to the soluble enzymes of the alimentary canal and intestinal flora (Wright *et al.*, 1965a).

B. Compounds that Are Rapidly Absorbed, Metabolized, and Completely Excreted

Mammalian metabolism of BHA was first investigated by Dacre *et al.* (1956) (see also Dacre, 1958, 1960). Those workers found that rabbits dosed orally with 1 g of the antioxidant excreted 46% as glucuronides, 9% as ether sulfates, and 6% as free phenols in the 24-hr urine. After a 0.5-g oral dose the recovery of glucuronides was 60% and of ether sulfates was 12%, and after a 0.25-g oral dose the recovery of glucuronides was 84% and of ether sulfates was 18%. Excretion of glucuronides in the urine was lowered by repeated dosage (0.5 g or 1 g) of BHA, and four or five daily doses of 1 g to a rabbit had cumulative and lethal effects. In experiments in which the elimination was not quantitative, the fate of the remainder of the dose was unknown.

From the urine of treated animals, Dacre *et al.* (1956) isolated the glucuronides of the two BHA isomers as barium (2-*tert*-butyl-4-methoxyphenylglucopyranosid) uronate and barium (3-*tert*-butyl-4-methoxyphenyl glucopyranosid) uronate.

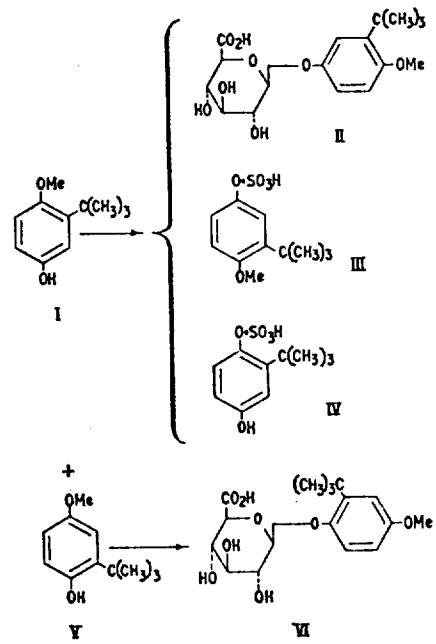
Dacre *et al.* (1956) found that when a rabbit was maintained for some weeks on a low-sulfur diet it showed no increase in ether sulfate excretion after administration of BHA, whereas administration of 1 g of Na_2SO_3 by stomach tube together with the BHA resulted in an excretion of 20% of the dose as ether sulfate. The proportion of administered BHA which was excreted as ether sulfate could therefore be varied experimentally.

In study of the metabolism of BHA in rabbits (Dacre *et al.* 1956), the dose was very large in comparison with human intake, which is probably less than 0.1 mg/kg. Since variations in dose may alter the proportions of metabolites excreted where more than one metabolic pathway is available (*cf.* phenol; Bray *et al.*, 1952), it might be argued that the metabolic pattern ought therefore to be studied with a range of doses including the lowest possible so that any unsuspected changes in metabolic pattern might be detected. These considerations prompted reinvestigation of the metabolism of BHA, and another animal species was chosen (Astill *et al.*, 1960).

In the rat, the metabolism of low doses of BHA resembled fairly closely that described (Dacre *et al.*, 1956) for the rabbit

for doses of 0.13–0.55 g/kg. At all the doses studied in the rat (Astill *et al.*, 1960), BHA and its component isomers were absorbed and readily metabolized. The main eliminative route was via the kidneys; fecal excretion in repeated-dose studies accounted for only 10.2% of the dose. For single doses of 0.002, 0.01, 0.025, 0.05, 0.10, and 0.40 g of BHA/kg, the 5-day recoveries were high (82–100% of dose). Excretion of unchanged BHA was 11% of a dose of 10 mg/kg, and 3–7% of a dose of 400 mg/kg, but the slightly higher proportion of unconjugated BHA excreted at the lower dose does not appear to be related to any tissue storage. It was concluded that at these low doses, BHA was largely absorbed and largely excreted; these doses approximate more closely to suspected human-use doses.

Astill *et al.* (1960) were unable to isolate the glucuronide (II) (see Scheme 1) of 2-*tert*-butyl-4-hydroxyanisole (I) (*cf.* Dacre *et al.*, 1956), but crude methyl O-triacetate, purified by column chromatography, was converted into 3-*tert*-butyl-4-methoxyphenyl-β-glucuronamide, identical to a synthetic specimen. The O-sulfate (III) of the 2-isomer was isolated as its Safranine bluish salt (Dodgson *et al.*, 1955), which was converted by cation



SCHEME 1. METABOLIC PATHWAYS OF BHA IN THE RAT

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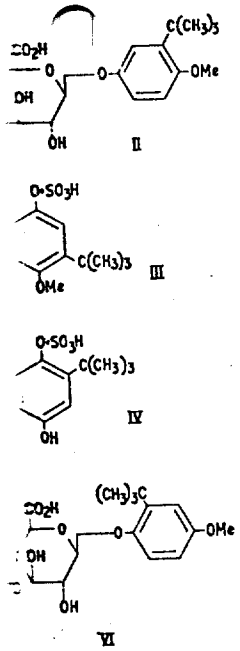

HWAYS OF BHA IN THE RAT

exchange into potassium 3-*tert*-butyl-4-methoxyphenyl sulfate; both salts were identical to synthetic specimens. The glucuronide (VI) of 3-*tert*-butyl-4-hydroxyanisole (V) was isolated as barium (2-*tert*-butyl-4-methoxyphenyl glucopyranosid) uronate from the glucuronide gum; no O-sulfate was isolated from the 3-isomer. The only other metabolite of BHA in rat urine was 2(or 3)-*tert*-butyl-4-hydroxyphenyl sulfate (IV), which gave SO_4^{2-} and *tert*-butylquinol on acid hydrolysis. A scheme for the metabolic pathways of BHA is shown in Scheme 1. 3-*tert*-Butyl-4-methoxyphenyl sulfate is the principal metabolite of 2-*tert*-butyl-4-hydroxyanisole, which was appreciably converted into the O-glucuronide when the sulfate pool was depleted.

When dogs were dosed with 350 mg of BHA/kg, the recovery in the excreta during 4 days was almost quantitative (Astill *et al.*, 1962); 62% of unchanged BHA was eliminated by the fecal route, because of nonabsorption (Astill *et al.*, 1962). Sulfate conjugation of the absorbed material in dogs occurred to the extent of 23% of dose. Separation of the O-sulfates as their phenazinium salts, followed by ion exchange and mild hydrolysis of the potassium salts, afforded 3-*tert*-butyl-4-hydroxyphenyl sulfate and a sulfate of a nuclear hydroxylation product of BHA. Excretion of unchanged BHA in the urine is in roughly the same proportion of the dose in dogs as in rabbits and rats. Only 5% of the dose was excreted as glucuronides, and glucuronide conjugation involved the 4-hydroxyl of BHA. The value of comparative metabolic studies is illustrated by the different metabolic pathway for BHA in dogs than in rabbits and rats.

When human volunteers ingested BHA (0.4–0.7 mg/kg), measurement of glucuronides in the urine established that the BHA absorbed was rapidly metabolized. Maximum excretion of glucuronides occurred within 17 hours of dosage, and excretion was complete within 48 hours of dosage. Subject to the errors of the analytical methods employed, 27–77% of the dose was excreted in the urine of men, as glucuronides. Free BHA was not detected in human urine, and there was little evidence for sulfate conjugation. This work (Astill *et al.*, 1962) shows that a major metabolic pathway for BHA is common to rabbits, rats, and man.

The possible storage of BHA in mammalian fatty tissues has also been investigated. When chickens were fed for 8 weeks and pigs for 4 months with 1000 ppm of BHA in their diet, no unchanged antioxidant was found in the dorsal and perinephric fats at the end of these feeding trials (François and Pihet, 1960).

Similarly, when groups of dogs were maintained for one year on diets containing BHA at 0.3, 3.0, 30, and 100 mg/kg as a 50% solution in propyleneglycol, there was no storage of BHA in the body fat, brain, liver, or kidney (Hodge *et al.*, 1964). Johnson *et al.* (1958) found that ingestion by rats of BHA in amounts equivalent to 100 and 500 times the concentration permitted in human food caused an increase in the stability of the perirenal fat, whereas administration of BHA at the normal level of use did not produce any significant increase in stability. In comparison with the amounts of antioxidant ingested by the rats, extraction of the fat depots afforded very small quantities of material with the antioxidant activity and color reactions of BHA. Whether BHA was directly responsible for the observed increase in stability of the fat was not proved. BHA may have stabilized the biological antioxidants and caused them to accumulate in the fatty tissues.

The foregoing studies on the metabolic fate of BHA were made with isotopically unlabeled antioxidant, and it is fair comment that the design of experiments and the resulting data were entirely dependent on the scope and limitations of the analytical methods employed. In this section, reference is therefore made to experimental detail.

When very small doses (97 μg) of [U-^{3}H]3-*tert*-butyl-4-hydroxyanisole were administered intraperitoneally to rats, more than 91% of the ^{3}H was excreted in the urine during 4 days (Golder *et al.* 1962). The rate of excretion of ^{3}H when rats were dosed with [U-^{3}H]3-*tert*-butyl-4-hydroxyanisole was similar to that of the metabolic products (Astill *et al.*, 1960) when unlabeled BHA and its component isomers were administered at dose levels ranging from 4 to 200 times as great. At a very low dose, BHA is probably excreted almost completely in a short time, and these results were considered to confirm the safety of BHA as a food antioxidant.

Parenteral administration of BHA and BHT to rats by Golder *et al.* (1962) deserves comment. Thus, the best method of making an intravenous injection of a lipid-soluble foreign compound is by slow infusion of a fatty emulsion or colloidal solution which has previously been equilibrated with plasma *in vitro*. Highly polar organic solvents are not ideal for this purpose, since they may cause considerable loss of material through penetration of the venous wall near the point of administration. Intraperitoneal administration of a lipid-soluble compound causes precipitation

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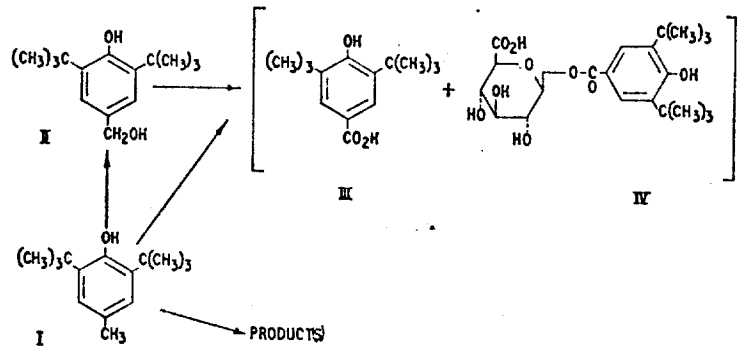
in the peritoneal cavity, with possible immobilization there of a variable proportion of the dose. This explanation may be relevant to the very variable proportion of a single intraperitoneal dose of [^{14}C]BHT which was excreted in the urine (Golder *et al.* 1962); elimination of ^{14}C in the feces was not, however, recorded.

The metabolism of Ionox 100 in the dog and rat was investigated by Wright *et al.* (1965b). A single dose (250 mg/kg) of [^{14}C] Ionox 100 (2,6-di-*tert*-butyl-4-hydroxy[^{14}C]methylphenol) administered orally to rats was excreted almost quantitatively in 11 days. Very little (0.29 ± 0.02%) of the dose was present in the carcass and remaining viscera after removal of the gut. There were two main eliminative routes, 15.6–70.8% of ^{14}C being excreted in the urine, and 75.2–27.0% in the feces during 11 days. During 96 hr, 12.5–66.9% of the ^{14}C was excreted in the urine and 60.8–24.3% in the feces. The big variation in the elimination pattern of individual animals suggested that the same primary metabolic product(s) is (are) eliminated primarily by the fecal and urinary routes. Approximately the same proportion of a single dose as that eliminated from rats during 96 hours was eliminated from dogs. Dogs and rats do not show a species difference in this pattern of elimination. In dogs, excretion of ^{14}C in the feces was the main eliminative route. After oral administration of [^{14}C] Ionox 100, the rate of elimination and recovery of radioactivity from animals did not seem to be affected by the size of the dose (250 mg/kg and 20 mg/kg were given to rats, and 17.62 mg/kg to dogs) or by the presence of triglyceride fat in the diet. Ionox 100 was completely metabolized in dogs and rats; unchanged Ionox 100 was absent from the urine and feces, and from the carcass when elimination was complete.

From the urine of rats treated with [^{14}C] Ionox 100, there were isolated 3,5-di-*tert*-butyl-4-hydroxybenz[^{14}C]oic acid and (3,5-di-*tert*-butyl-4-hydroxybenz[^{14}C]oyl-β-D-glucopyranosid) uronic acid, which was similarly prepared from the urine of rabbits dosed with 3,5-di-*tert*-butyl-4-hydroxybenzoic acid. The crude reaction product from successive methylation and acetylation was purified by column chromatography to give methyl (3,5-di-*tert*-butyl-4-hydroxybenz[^{14}C]oyl tri-O-acetyl-β-D-glucopyranosid) uronate, identical with material similarly obtained from the metabolic product of 3,5-di-*tert*-butyl-4-hydroxybenzoic acid in rabbits. 3,5-Di-*tert*-butyl-4-hydroxybenz[^{14}C]oic acid accounts quantitatively for the ^{14}C in extracts of the feces. In rats, 3,5-di-*tert*-butyl-4-hydroxybenzoic acid accounts for 50–85% of a dose of Ionox 100, and (3,5-di-*tert*-butyl-

4-hydroxybenzoyl-β-D-glucopyranosid) uronic acid for 47–10%; in dogs, the unconjugated acid accounts for 85% and the ester glucuronide for 10–12%. 3,5-Di-*tert*-butyl-4-hydroxyhippuric acid, which was synthesized, is not formed *in vivo*. Other metabolites detected in small quantity in the feces and urine of animals dosed with Ionox 100 have not been identified.

In this work, 3,5-di-*tert*-butyl-4-hydroxybenzoic acid (III), Scheme 2, and the related ester glucuronide (IV) were shown to be major metabolites of the structurally related antioxidants BHT (I) and Ionox 100 (II) in rats. The elimination of Ionox 100 metabolites from rats is faster than that of BHT and its metabolites. Unlike BHT, unchanged Ionox 100 could not be detected in the bodies of these animals.



SCHEME 2. SCHEME FOR THE METABOLISM OF BHT (I) AND IONOX 100 (II) IN RATS

After publication of our results on the metabolism of Ionox 100 (Wright *et al.*, 1965b), our attention was drawn to work by Akagi and Aoki (1962a) in which it was claimed that 3,5-di-*tert*-butyl-4-hydroxybenzaldehyde, 3,5-di-*tert*-butyl-4-hydroxybenzoic acid, di-(3,5-di-*tert*-butyl-4-hydroxybenzyl), an unidentified glucuronide (not [3,5-di-*tert*-butyl-4-hydroxybenzoyl-β-D-glucopyranosid] uronic acid, and unchanged Ionox 100 had been isolated from the urine of rabbits dosed with Ionox 100 (1.6 g/kg body weight).

Identification of the free acid agrees with findings of Wright *et al.* (1965b) for rats, and it is rather surprising that the corresponding ester glucuronide was not also identified, but it would have been hydrolyzed in the exhaustive extraction with ether if several changes of solvent were not used and if the ether extracts

in animals with the highest concentration of Ionox 330 (31600 ppm) in their diet. This growth suppression seems to be due to dietary intake and was not interpreted as a toxic effect by Stevenson *et al.* (1965).

2. BHA

a. Acute Toxicity. When BHA was administered orally, the LD_{50} values (expressed as mg/kg body weight) were 2000 (mice) and more than 5000 (rats) (Bunnell *et al.*, 1955; Lehman *et al.*, 1951).

b. Short-Term Studies. In a half-year feeding study with groups of rats on various dietary levels of BHA, animals on the 3% level did not eat enough to gain weight and had to be put onto the 2% diet for a time and then returned to the 3% regimen. Even at the 2% level, food consumption was below optimum. No effects due to the food additive were revealed by histopathological examination (Wilder and Kraybill, 1948). Combination of BHA with other food additives at fifty times the levels normal in bread had no adverse effects when fed (in bread) to groups of rats for 32 weeks (daily doses of BHA ranged from 3.3 to 7.0 mg/kg body weight) (Graham *et al.*, 1954; Graham and Grice, 1955). When rats were fed with BHA at 500 and 600 mg/kg body weight for 82 and 68 days, respectively, no behavioral changes were observed, but a reduction was found in growth rate and in the activities of blood catalase and peroxidase. BHA caused increased relative liver weight in rats (Karplyuk, 1959).

Daily oral administration of BHA (1 g) to rabbits for 5–6 days caused a tenfold increase in the excretion of Na^+ and a 20% increase in the excretion of K^+ in the urine. A decrease in extracellular fluid volume approximately restored the concentration of plasma Na^+. Serum K^+ fell after 5 days of treatment, and in muscle cells, K^+ was being replaced with Na^+; changes in heart muscle followed those in skeletal muscle but were not as severe. BHA may have a direct action on the kidneys; there were some changes in the adrenal cortex, and increased excretion of aldosterone occurred together with loss of Na^+ and K^+ (Denz and Llaurado, 1957).

When BHA was fed to groups of dogs for one year at various dietary levels (0.3, 3.0, 30, and 100 mg/kg body weight/day) in propylene glycol, there were no ill effects; renal function, hematology and histopathology of the main tissues, and organ weights were normal (Hodge *et al.*, 1964).

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c. Long-Term Studies. BHA fed to groups of rats for 22 months at various dietary levels caused no ill effects. Weight gains were comparable in all groups, reproduction was normal, and young rats fed on the same diet grew normally. The litters were strictly comparable, for all animals of all groups, with regard to number, size, weight, weight gain, and mortality. No changes due to this antioxidant were revealed by histopathological examination (Wilder and Kraybill, 1948). Similar tests were made with an additional group of rats fed for 21 months with a diet containing 0.12% BHA and supplemented with 2% lard. Histopathological examination, again, revealed no changes, and animals fed BHA were identical to the controls (Wilder and Kraybill, 1948). Brown *et al.* (1959) fed BHA to rats for 2 years at various dietary levels, and in some of the animals on the highest (0.5%) level there was a slight reduction in mature weight and increased relative liver weight. There were no effects on: reproductive cycle; histology of kidneys, liver, skin, and spleen; relative weights of heart, kidneys, and spleen; or mortality. The toxicity of BHA was unaffected by the dietary load of fat.

Wilder *et al.* (1960) fed BHA for 15 months at various levels to groups of Cocker Spaniel pups, and found that throughout this period the general health and weight gains of the dogs were good. Hemoglobin and blood-cell counts were not affected significantly at the dietary levels of antioxidant used. Histopathological examination showed no changes other than normal variation except that three of the animals that had received the highest dose (250 mg/kg body weight/day) suffered liver damage.

Subacute toxicity data for BHA are satisfactory with regard to the numbers of species tested. Tests on BHA have taken into consideration the possibility that the toxicity of antioxidants may be changed at the temperatures used in food processing.

3. BHT

a. Acute Toxicity. When BHT was administered orally, the LD_{50} values (expressed as mg/kg body weight) were 1700–1970 (Deichmann *et al.*, 1955), 2450 (Karplyuk, 1960) (rat), and 2000 (mouse) (Karplyuk, 1960); and the LD_{100} values were: 3500 (rat), 2500 (mouse) (Karplyuk, 1960), 940–2100 (cat), 2100–3200 (rabbit), and 10700 (guinea pig) (Deichmann *et al.*, 1955).

b. Short-Term Studies. Weanling rats of each sex were chronically fed diets containing 10 and 20% lard supplements with 0.0001, 0.1000, or 0.5000% BHT added, and the 0.5% BHT caused

increased concentrations of serum cholesterol and phospholipid within 5 weeks. Female rats fed for 8 months on a diet containing 10% lard and 0.1% BHT showed increased serum cholesterol levels (Day *et al.* 1959). Brown *et al.* (1959) studied the effect on rats of BHT (and BHA) at levels up to 0.5% in a diet containing 10 or 20% added fat. BHT compared unfavorably with BHA in the extent to which it diminished initial growth rate and increased relative liver weight. Loss of hair from the heads of rats was claimed with diets containing 0.1% BHT or more; this was enhanced by increasing lard concentrations in the diet. Three of thirty rat litters contained young born without eyes when the parents had been fed 5 months on diets containing BHT, and it is relevant that anophthalmia was not observed in any of the stock or other experimental animals.

Deichmann *et al.* (1955) had previously reported that rats chronically fed diets containing BHT found 0.5 or 1.0% of the antioxidant unpalatable. The animals could be conditioned to ingest such foods if these concentrations were attained gradually. Paired feeding experiments with groups of 5 or 10 rats for 25 days demonstrated that diets containing 0.8 or 1.0% BHT depress food intake, and that a concentration of 1.0% of BHT in the diet retards weight gain.

In another exploration of the safety evaluation of BHT, Karplyuk (1959; 1960) fed rats for two or three months with BHT, BHA, or propyl gallate, equivalent to 0.3% of the diet, dissolved in lard. Judged to be the most toxic of these antioxidants was BHT. This antioxidant alone affected phospholipid synthesis in liver, and the hydrolysis and transport of neutral fat from the liver. Only BHT decreased the activities of three plasma enzymes—catalase, cholinesterase, and peroxidase—whereas BHA reduced catalase and peroxidase activities, and propyl gallate reduced cholinesterase and peroxidase activities.

Sporn and Schöbesch (1961) also concluded that BHT had a toxic effect. They also considered that the low margin of safety of BHT made it undesirable as a food additive. Those workers used groups of 16 white rats fed diets containing 9.6, 16.6, and 19.9% of casein, and the test groups also received 0.2% of BHT in their diet. In each case, BHT improved growth and protein efficiency. Liver nitrogen content was greatly reduced in BHT-treated animals unless the concentration of BHT in the diet was only 0.02%. It was also alleged that after an unspecified period of fasting, the recovery of liver protein was impaired in rats chronically fed

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0.2% BHT. Liver lipids increased at the higher dietary level of BHT, but not at 0.02% BHT. Further, the increased ascorbic acid content of the adrenal medullae of rats fed 0.2% BHT was considered indicative of stress imposed by BHT. The present author, however, claims that when ascorbic acid is expressed in terms of adrenal concentration (μg/g of tissue), it becomes clear that no significant increase in concentration has occurred.

Finally, under short-term studies with rats, mention ought also to be made of work of Gaunt *et al.* (1965a) in which groups of 48 rats were chronically administered diets containing 0.1% BHT or BHA. Measurements of body growth, food consumption, and organ weights, together with detailed histological examination, showed no differences from the control group. During 16 weeks, increases occurred in relative liver weight and in relative adrenal weight, without histopathological evidence of damage. Histochemical observations on glucose-6-phosphatase and glucose-6-phosphate dehydrogenase again showed no differences from the control group. The findings of Golberg and co-workers concerning changes in liver enzyme equipment caused by BHT have been discussed (Section V,D); reference might be made here to their reversibility during recovery of the animals.

In rabbits, acute effects on electrolyte excretion similar to those described for large doses of BHA (Section VI,A) were also obtained after administration of 500–700 mg of BHT/kg body weight (i.e., about 2% in the diet). At lower doses these effects were not encountered (Denz and Llaurado, 1957).

Deichmann *et al.* (1955) induced severe diarrhea in a group of four dogs fed doses of 1.4–1.7 g of BHT/kg body weight every 2–4 days for 4 weeks. No gross histopathological changes were revealed post-mortem. Dogs fed 0.17–0.94 g of BHT/kg body weight 5 days a week for 12 months showed no symptoms of intoxication and no histopathological changes post-mortem.

As has been stated (Section V,C), fertility, hatchability of eggs, and health of chicks did not differ from those of a control group when 10 laying hens were fed 0.125% BHT for 34 weeks (Shellenberger *et al.*, 1957).

c. Long-Term Studies. Deichmann *et al.* (1955) found that, when groups of 15 male and 15 female rats were given diets containing 1% lard and 0.2, 0.5, or 0.8% BHT for 2 years, they showed no symptoms of intoxication and no histopathological changes post-mortem. In an experiment designed to take into account the possibility that the toxicity of the antioxidant (0.5%

BHT) may be changed under conditions used in food preparation (150°C for 30 min), Deichmann *et al.* (1955) showed that there were no effects on weight gain or on the hematopoietic system; histopathological studies were negative. Ingestion of 1% BHT by male and female animals caused subnormal weight gain and increased relative liver and brain weights, but histopathological examinations were negative for this group as well. At these levels of dose, BHT did not affect the hematopoietic system. In all of these experiments a number of rats died, but deaths were not interpreted as being related to the concentration of antioxidant ingested (Deichmann *et al.*, 1955).

When rats were chronically fed diets containing 0.5% BHT, Brown *et al.* (1959) found no adverse effects on reproductive cycle, structure of kidneys, liver, skin, and spleen, or relative weights of heart, kidneys, or spleen. When rats were fed a diet containing 0.1% BHT and 10% hydrogenated coconut oil for 2 years, mortality was not increased.

Karplyuk (1960) administered 0.2% BHT (or BHA) in lard at a dose level of 4 mg/kg body weight/day for 6 months, followed by 8 mg/kg body weight/day for an additional 6 months. At the end of the first 5 months, the rats were mated. The 1-month-old young were separated and put on a regimen of 8 mg of BHT/kg body weight/day. After another 6 months, a third generation was produced by the second, and treated with the same BHT regimen for 6 months. All three generations were examined for weight gain. The hematopoietic system, serum albumin, blood cholesterol, nonprotein nitrogen, blood enzymes (catalase, cholinesterase, and peroxidase), Takata-Ara reaction, and Weltmann coagulation test were investigated. Relative liver weight, dry weight, and cholesterol, fat, glycogen, and phosphorus contents of livers were determined. Histopathological examinations were made of the brain, liver, and several other organs; and the numbers of young, their weights, appearance, and age when eye-opening occurred were recorded. Rats treated with BHT (and BHA) were normal in all these respects.

In a later paper (Karplyuk, 1962), milk and meat were withdrawn for 14-day periods from the diets of animals committed to the long-term feeding regimen with BHT (Karplyuk, 1960) during the seventh and eleventh months of test; body weights of test and control animals followed similar patterns.

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similar patterns.
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study of Frawley *et al.* (1965b) should be mentioned (see also Section V,C). At 100 days, sixteen females at each dietary level (300, 1000, or 3000 ppm BHT) were mated with animals belonging to the same dietary level. Examined in mothers were fertility, parturition, and lactation; examined in the young were survival, viability, and growth. Ten days after weaning, the females were mated again, and similar observations were recorded for mothers and young. The following clinical tests were made on the first generation: full blood count, cell indices, reticulocyte count, prothrombin time, serum total cholesterol, cholesterol esters, GOT, GPT, alkaline phosphatase, fasting blood sugar, blood urea nitrogen, icterus index, urinary reducing substances, albumin, and microscopic elements. All clinical tests on the first generation were repeated on the second generation, together with the following additional clinical tests: serum lipids, phospholipids, mucoproteins, α- and β-lipoproteins, cholinesterase, erythrocyte and cerebral acetylcholinesterase, adrenal ascorbic acid, cholesterol, and cholesterol esters. Complete histopathological examinations were made after the second litter was weaned. In the first and second generations, thirty organs and tissues were examined histologically. After the second litter was weaned, these offspring were fed the same diet as their parents until they were 100 days old, when they were mated according to the accepted practice. The same clinical and pathological observations were recorded for these animals and the two litters of young.

At the level of 3000 ppm BHT, growth gain of parents and young was depressed 10–20%. After 28 weeks, there was a 20% increase in serum cholesterol over controls, and after 42 weeks a 10–20% increase in relative liver weight. The remaining observations at the 3000-ppm level, and all observations at the 300- and 1000-ppm levels, were strictly comparable with those of controls. Throughout the trial, reproduction was normal and no teratogenic effects were noticed.

The only long-term study with a nonrodent species was made by Karplyuk (1960). He fed pairs of dogs for one year with fat containing 4% BHT (or BHA) at a dose level of 4 mg of antioxidant/kg body weight/day. No toxic effects were found; results cited refer particularly to liver dry weight, glycogen, lipid phosphorus, and cholesterol.

Subacute toxicity data for BHT are satisfactory with regard to the numbers of species tested, especially in the short-term studies.

Tests on BHT have taken into consideration the possibility that the toxicity of antioxidants may be changed at the temperatures used in food processing.

B. Relationship of Metabolic Fate and Related Aspects of Physiological Chemistry to Evaluation of the Safety-in-Use of Food Additives

If a substance produces no toxic symptoms in toxicological testing, the fate of a dose may be traced to provide convincing evidence of its harmlessness (see Section I,A). Commensurate with this supposition, investigations with Ionox 330 have yielded very promising results. Thus, no toxic symptoms were detected in a short-term study with rats (Stevenson *et al.*, 1965), and, in an investigation of the fate of Ionox 330 (Wright *et al.*, 1965a), nonabsorption was demonstrated in pigs and rats; unchanged antioxidant was quantitatively recovered from the 48-hour feces of rats, dogs, and man. For a full safety evaluation, subacute toxicological testing would have to include work with a nonrodent species and a long-term study that might incorporate some carcinogenicity testing. Since no symptoms would be expected, a feeding test with humans could be conducted.

BHA has for long enjoyed the reputation of being a safe food additive (see World Health Organization, 1962a), and extensive toxicological testing has not exposed significant toxic symptoms. The data on toxicity to animals are satisfactory with regard to numbers of animals and species, but the duration of long-term tests with nonrodent species might be open to criticism. The experimental observations allow for the effect of heat on the antioxidant and for the possibility that other additives in the diet may potentiate the toxicity of BHA. Metabolism studies have shown that single doses of BHA are absorbed, metabolized, and excreted in a short period (Section III,B). These results and the absence of toxic symptoms during toxicological testing are considered to vindicate the safety of BHA as a food additive (see Section I,A).

Further, on account of the conjugation of BHA isomers in the liver, some accompanying variation would be expected in the rate of liver enzyme synthesis-repression of some enzymes and induction of the formation of others. In fact, enhanced activities of microsomal drug-metabolizing enzymes rapidly returned to normal when the animals were allowed to recover. At high doses, BHA caused neither changes in the activities of other smooth endoplasmic reticular enzymes, glucose-6-phosphatase and glucose-6-phos-

dded)

m	
erence, B—A	Calcd Equiv. % Ipronidazole
—0.003	+0.00007
—0.007	+0.0002
—0.002	—0.00005
—0.012	+0.0003
—0.004	—0.0001
—0.002	—0.00005
—0.007	+0.0002
—0.008	+0.0002

plus zinc bacitracin
arter plus chlortetra-
iler starter plus pro-
omycin (50 g/ton);
nilic acid (50 g/ton);
4-hydroxy-3-nitro-
on); turkey starter
s showed any direct
e only peak visible on
t of ipronidazole. In
re el d for at least

of the spectrophoto-
ng the sample blank,

erence	% Recovery
0.209	98
0.210	98
0.202	95
0.215	100
0.213	100
0.213	100
0.207	97
0.215	101
0.204	95
0.214	
0.213	

d 0.012% ipronidazole

pronidazole, 0.012%

mple bs.	Net Abs.	% Recovery
.328	0.310	97
.341	0.320	100
.335	0.317	99
.325	0.309	97
.331	0.312	98

different types of feeds containing various drugs but no ipronidazole were assayed. Table 2 shows the absorbance readings obtained at 318 ± 1 nm. Small differences between absorbance readings before and after evaporation establish the validity of the determined sample blanks.

Ipronidazole, at 600 μg, was added to 10 g unmedicated feed. Medicated and unmedicated feeds were assayed by the above procedure. Absorbances were obtained at 318 ± 1 nm and are summarized in Table 3. Average differences between the second and third columns are negligible, indicating complete removal of ipronidazole by 10 min evaporation at 75 ± 2°C.

Ipronidazole was added to 10 g control feed as 600 μg in 10 ml 0.2*N* HCl. The mixture was allowed to stand 5 min, after which 90 ml 0.2*N* HCl (40°C) was added and the sample was assayed. Average recovery (Table 4) of ipronidazole added to feed was 98%. Similar tests were carried out with a turkey starter ration to check both recovery and reproducibility at three different drug levels. Five separate assays were run at each level. The results are summarized in Table 5. Again recoveries averaged 98%. The pooled standard deviation was ± 2%.

This paper was presented at the 83rd Annual Meeting of the AOAC, Oct. 13–16, 1969, at Washington, D.C.

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FOOD ADDITIVES

GLC Determination of Butylated Hydroxyanisole and Butylated Hydroxytoluene in Breakfast Cereals

By DAVID M. TAKAHASHI (Food and Drug Administration, 50 Fulton St., San Francisco, Calif. 94102)

Eighteen laboratories studied the AOAC official first action method for GLC determination of BHA and BHT in corn and rice ready-to-eat breakfast cereals. Recoveries obtained were superior to those from the previous collaborative study. Corn cereal spiked with 29.5 ppm BHA and 30.6 ppm BHT gave 98 and 101% average recoveries, respectively. Rice cereal spiked with 25.2 ppm BHA and 25.6 ppm BHT gave 98 and 100% average recoveries, respectively. The method, as amended, is recommended for adoption as official final action.

The AOAC official first action method for the gas chromatographic (GLC) determination of butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) in corn and rice ready-to-eat breakfast cereals (1) was subjected to an extensive collaborative study involving 18 laboratories. Results indicate that nearly all the participants recovered antioxidants added to both cereals and the data obtained by this study were superior to those obtained by the previous collaborative study (2).

Collaborative Study

Preparation of Collaborative Samples

Corn and rice breakfast cereals were ground to pass a No. 20 sieve and mixed well. The air in all containers for cereals, standards, and spike solutions was displaced with nitrogen to prevent loss

This report of the Associate Referee was presented at the 83rd Annual Meeting of the AOAC, Oct. 13–16, 1969, at Washington, D.C.

of antioxidants. Spike solutions A and B contained BHA and BHT in 2,2,4-trimethylpentane. Collaborative samples were shipped by air mail without Dry Ice.

Instructions to Collaborators

Each collaborator was furnished with 100 g each of ground and mixed corn and rice cereals, designated as cereals A and B, respectively; one vial each of BHA (3-isomer), BHT, and di-BHA standards; and two sealed glass ampules containing spiking solutions, also designated as A and B. Collaborators were asked to make single determinations of the antioxidant content of cereals A and B before and after spiking with the unknowns.

Collaborators were instructed to use the AOAC official first action method (1), with a few minor modifications. (The revised method is given below.) They were asked to report the antioxidant content on a ppm basis and to submit the calculated results, worksheets, and GLC charts.

Directions for analysis were as follows: *Practice run* (analyst who is familiar with this method may omit this): Using 20.0 g cereal A, make a single recovery determination at the 10 ppm levels of BHA and BHT. Evaporate under nitrogen to <20.0 ml and dilute to 20.0 ml before GLC injection. Usual recovery range: 90-110%. Determine the antioxidant originally present in cereals A and B. Dilution of extract to 10.0 ml final volume is satisfactory in these samples.

Spiked run: Spike by adding 2.00 ml unknown A to 20.0 g cereal A packed in a chromatographic column. Let the liquid sink into the cereal, top with glass wool plug, and proceed with the analysis. Evaporate sample under nitrogen to <25.0 ml and dilute to 25.0 ml. Similarly, spike 20.0 g cereal B with 2.00 ml unknown B.

The following precautionary notes in antioxidant analysis were also given to the collaborators: (*1*) Since BHA and BHT continue to break down in food, analyses of these antioxidants should be conducted immediately upon receipt of sample. If this is not possible, the sample should be stored frozen until time for analysis. (*2*) Crystalline standards will keep almost indefinitely without loss of potency or any significant discoloration, when stored in a cool dark place under nitrogen. The unknown spike solutions should also be stored in a cool dark place. (*3*) The control and the unknowns should be analyzed on the same day.

METHOD

Principle

BHA and BHT are extd from ready-to-eat breakfast cereals with CS_2 and detd by GLC, using flame ionization detection.

Apparatus

(a) *Gas chromatograph.* — Barber-Colman Co. Model 5000, or equiv., with H flame ionization detector and strip chart recorder. *Operating conditions:* temps—column 160°, detector 210°, flash heater 200°; N flow rate, to elute BHT in 3–4 min from QF-1 column and elute BHA in 3–4 min from Apiezon column; H flow rate, ca 40 ml/min for Apiezon and ca 25 ml/min for QF-1; air flow rate, ca 340 ml/min; electrometer sensitivity, 500× (5×10^{-10} amp full scale deflection) with 1 mv recorder. Adjust H and air flow rates if necessary. Alternatively, adjust electrometer sensitivity so 0.1 μg BHA gives ca 50% deflection. Repeat injections until constant peak hts are obtained on succesive injections of identical vol. of std mixt.

Order of appearance from Apiezon column (4′): BHA, BHT, di-BHA. Order of appearance from QF-1 column (6′): BHT, BHA, di-BHA.

Use of 2 GLC columns serves to identify BHA and BHT. Use Apiezon L/Gas Chrom Q, 4′ column to resolve 2- and 3-isomers of BHA. Adjust GLC parameters to obtain good sepn of BHA isomers, such as adjusting column temp. to 150° and electrometer sensitivity to 1000×. Adjust amt BHA std injected to give 50% deflection.

(b) *GLC columns.*—Glass, 4′ × 4 mm and 6′ × 4 mm, packed with Apiezon L and QF-1 silicone oil (Fluoro silicone fluid, FS 1265), resp., on 80–100 mesh Gas Chrom Q (Applied Science Laboratories). Prep. columns and column material by carefully washing insides of 4′ and 6′ columns and small amt fine glass wool with dichlorodimethylsilane soln, rinsing with MeOH, and drying.

Slowly sprinkle ca 50 g Gas Chrom Q into 800 ml beaker almost filled with CCl_4. Remove fine particles that remain on surface with vac. line and trap. Decant solv. and dry GLC support.

Transfer 20.0 g dried Gas Chrom Q to 500 ml r-b flask. Add 100 ml $CHCl_3$ or CH_2Cl_2 and mix gently. Dissolve 1.0 g Apiezon L or 2.0 g QF-1 in 50 ml $CHCl_3$ or CH_2Cl_2, add to flask, and mix gently. Evap. to dryness, using rotary vac. evaporator and H_2O bath (35° for CH_2Cl_2 and 70° for $CHCl_3$).

Carefully plug exit of column with small plug of fine glass wool and thru-hole septum. Apply vac. to exit port and slowly add coated support (Apiezon L/Gas Chrom Q for 4′ column; QF-1/Gas Chrom Q for 6′ column) thru injection port, tapping very gently to aid compaction. Pack to within 1 cm of

area heated by flash heater. Plug with fine glass wool and condition ca 3 days at 200° with slow stream of N (ca 10 ml/min) or until steady baseline is obtained.

(c) *Chromatographic tube.*—25 × 200 mm glass tube with small drip tip (4 mm id, 6 mm od × 50 mm long), with or without medium porosity fritted disk, with close-fitting tamping rod.

(d) *Fine glass wool.*—Wash with CS_2 and dry.

Reagents

(a) *Carbon disulfide.*—Reagent grade; nearly colorless. If distinctly yellow, distill before use.

(b) *BHA, BHT, and di-BHA std mixture.*—0.02 μg/μl each of BHA, BHT, and di-BHA in CS_2. Dissolve 1.00 mg each of BHA and BHT in small amt CS_2, add 10.0 ml internal std soln, and dil. to 50 ml with CS_2 or prep. by dilu of more concd solns with CS_2. Prep. fresh and store in low-actinic glassware.

(c) *Internal std soln.*—0.1 μg di-BHA/μl CS_2. Prep. fresh and store in low-actinic glassware.

(d) *Dichlorodimethylsilane soln.*—Dil. 5 ml to 100 ml with toluene.

Determination

(Protect all solns from light; complete assay in 1 day. Use either GLC column for analysis.)

Grind sample to pass No. 20 sieve and mix well. (When necessary, ground sample may be stored frozen under N for few days.) Place, if necessary, small plug of fine glass wool at bottom of chromatgc tube, add 20.0 g sample to column, using tamping rod to pack it firmly without solv., and top column with another small glass wool plug, tamped down. Mark 100 ml beaker at 50 ml level and place under column to collect eluate. Add three 5 ml portions of CS_2 to column, letting each portion sink into column before adding next. Elute CS_2 at ca 5 ml/min; use N, if necessary, to maintain flow rate.

Add several 10 ml portions of CS_2, letting each portion sink into column until 50 ml eluate collects. Rinse tip of column with small amt CS_2. Accurately add di-BHA to eluate to obtain concn after evapn of 0.02 μg di-BHA/μl final soln. Evap. eluate under gentle stream of N in hood at room temp. to small vol. (<5.0 ml). Accurately dil. evapd sample to appropriate vol. (e.g., 5.0 ml) for GLC analysis. Inject 3.0-9.0 μl sample, using 10 μl syringe, into gas chromatograph. Before and after each series of sample chromatograms, inject 3.0-9.0 μl std mixt. and average std values for calcns. Measure each peak ht in mm. (Ht of BHA, BHT, and di-BHA peaks should be in range of 30-95% full scale deflection.)

Calc. ppm antioxidant present, correcting for internal std, as follows:

ppm BHA or BHT = $(H_x/H_s) \times (C_s/C_x) \times (H_{xi}/H_{si}) \times (C_{xi}/C_{si})$, where H_x and H_s = ht (mm) of sample and std peaks, resp.; H_{xi} and H_{si} = ht (mm) of internal std peaks in sample and std, resp.; C_x and C_s = concn of sample (g/μl) and std (μg/μl), resp.; and C_{xi} and C_{si} = concn (μg/μl) of internal std in sample and std solns, resp.

Results and Discussion

Excellent results were obtained by this collaborative study (Tables 1-3). The data indicate better recovery than those obtained by the previous collaborative study (2). Nevertheless, the unstable nature of antioxidants in food must fully be recognized to insure continuously accurate and reliable analyses of these compounds. For example, pronounced decomposition of antioxidants in the original sample occurred when it was left at room temperature for a prolonged period of time. Prepared ground cereals must be stored frozen until analysis to avoid loss of antioxidants in sample. Once the analysis is started, however, the entire operation should be conducted as smoothly and rapidly as possible.

Table 1. Recovery of antioxidants in practice run using corn cereal (known amount of antioxidants spiked by collaborators)

	Added, ppm		Recovered, ppm		Recovery, %	
Coll.[a]	BHA	BHT	BHA	BHT	BHA	BHT
1	11.3	10.4	10.5	10.9	93	105
2	10.0	10.0	10.0	9.7	100	97
4	10.15	10.08	9.84	10.21	97	101
5	10.24	10.10	9.4	9.4	92	93
6	9.65	9.58	9.79	9.53	101	100
8	10.26	9.76	10.88	9.79	106	100
9	10.02	10.04	9.75	9.52	97	95
10	10.3	10.1	10.2	10.71	99	106
13	10.3	10.1	10.44	9.58	101	95
14[b]	10.3	10.3	10.07	9.47	98	92
15	11.2	12.1	11.25	13.91	100	115
16	10.35	10.32	10.2	10.0	99	97

[a] Collaborators 3, 7, 11, 12, and 17 did not analyze a practice sample; Collaborator 18 obtained about 50% recovery for BHA and about 100% for BHT.
[b] Rice cereal used for sample.

GLC Columns

Twelve collaborators used the Apiezon L column, five the QF-1 column, and one 10% DC-200 on 80-100 mesh Gas Chrom Q. Five collaborators successfully used the 5% load of Apiezon L column; four collaborators also successfully used the 10% load of QF-1 column.

Collaborator 3 reported that his Apiezon L column developed brown spots after conditioning and that he obtained poor results for injections of

Table 2. Recovery of antioxidants from corn cereal spiked with 29.5 ppm BHA and 30.6 ppm BHT

Coll.[a]	ppm BHT in Original Sample	Recovery, ppm		Recovery, %	
		BHA	BHT	BHA	BHT
1	8.6	28.9	30.4	98	99
2	10.4	29.7	34.5	101	113
3	8.5[b]	29.5	30.7	100	100
4[c]	10.3	29.1	29.9	99	98
5	9.8	30.3	33.2	103	108
6	9.8[d]	26.9	27.6	91	90
7	6.8[e]	29.0	32.8	98	107
8	10.9	30.4	31.6	103	103
9	10.1	28.7	31.0	97	101
10	9.8	28.5	30.0	97	98
11	10.4	30.0	31.1	102	102
12	8.7	27.8	31.0	94	101
13	8.9	28.7	28.4	97	93
14	9.5	28.6	30.6	97	100
15	9.6	30.3	28.5	103	93
16	1.2[f]	26.8	30.3	91	99
17	10.4	29.3	31.8	99	104
Av.		29.0	30.8	98	101
Std dev.		1.1	1.7	3.7	5.7
Av. std dev.		0.26	0.42	0.9	1.4

[a] In the trial run, Collaborator 18 could recover BHT but not BHA and therefore did not analyze the collaborative sample. The reason for the low recovery of BHA is unknown.
[b] Sample left at room temperature for 12 days.
[c] 10% DC-200/Gas Chrom Q used for determination.
[d] Sample left at room temperature for 5 days.
[e] Sample left at room temperature for about 15 days.
[f] Analysis made about 4 months after collaborative sample preparation.

standard mixtures; he obtained better results with the QF-1 column.

The QF-1 column did not work for Collaborators 4 and 15; Collaborator 15 changed to the Apiezon L column with success, while Collaborator 4 obtained good recoveries with 10% DC-200 on 80-100 mesh Gas Chrom Q. This latter column produced a small solvent peak and good resolution of BHA and BHT peaks; the order of elution of antioxidants is identical with that of the Apiezon L column: BHA, BHT, di-BHA.

Collaborator 9 prepared his Apiezon L column using a different method (3), but his results were comparable.

Collaborator 11 used the original Apiezon L column he used in the 1967 collaborative study (2). Although the resolution of BHA and BHT was not as good as in the initial study, it did not interfere with the determination of antioxidants.

Collaborator 13 used a 4′ × 2 mm id 10% QF-1/Gas Chrom Q column. It was necessary to use a column temperature of 145°C and to adjust all gas flows to achieve good sensitivity and resolution of BHA and BHT.

Collaborator 14 could not get an even coating of Apiezon L. After noticing black specks in the Gas Chrom Q he used, he prepared a column of 10% QF-1 on Chromosorb W-HP (similar in performance to Gas Chrom Q in pesticide analysis). Good results were obtained.

Determination

Two collaborators used glass columns with a coarse porosity fritted glass disk. Others used columns either with medium porosity fritted glass disks or with a glass wool plug.

Six collaborators reported the use of 1 mv recorders. Satisfactory results were obtained with all the GLC instruments used in this study: Barber-Colman Model 5000, Barber-Colman Model 5000 with Coleman-Hitachi No. 165 recorder, F&M Model 5750, Varian Aerograph Model 2100, and Packard Model 7621.

Collaborator 8 optimized the flow rate and adjusted the column temperature to achieve proper retention time. He encountered no other problem.

Additional comments from Collaborator 11 were as follows: Assay and calculations were easily completed within a normal 8 hr working

Table 3. Recovery of antioxidants from rice cereals spiked with 25.2 ppm BHA and 25.6 ppm BHT

Coll.[a]	ppm BHT Original Sample	Recovery, ppm		Recovery, %	
		BHA	BHT	BHA	BHT
1	11.5	24.4	26.2	97	102
2	12.3	24.7	28.4	98	111
3	13.3[b]	25.6	25.0	102	98
4[c]	13.3	25.3	24.5	100	96
5	10.1	23.9	23.9	95	93
6	11.0[d]	25.9	26.9	103	105
7	10.0[e]	24.6	23.8	98	93
8	11.6	25.3	25.8	100	101
9	5.6[g]	26.1	19.6[g]	104	77[g]
10	10.6	25.9	27.6	103	108
11	12.0	25.5	26.0	101	102
12	10.6	22.8	24.3	90	95
13	10.5	24.9	24.7	99	96
14	11.2	24.6	25.7	98	100
15	10.9	23.5	24.4	93	95
16	4.3[f]	22.9	26.0	91	102
17	11.6	25.4	26.2	101	102
Av.		24.8	25.6	98	100
Std dev.		1.0	1.3	4.1	5.2
Av. std dev.		0.25	0.33	1.0	1.3

[a–f] See footnotes, Table 1.
[g] Reason for low value is unknown; excluded from calculations as outlier.

day. Flow rates from the glass wool-plugged columns were about 5 ml/min. The method is rapid, direct, and ideally suited for cereal foods. No difficulties were encountered with this method.

Collaborator 14 reported that water condensation at the evaporation step caused a problem because of the high humidity in his laboratory. He solved the difficulty by placing the beaker containing the CS_2 extract in a pan of warm water and then directed a nitrogen stream over the surface of the CS_2 solution.

Collaborator 18 was unable to obtain more than 50% recovery of BHA in the trial run. BHT recovery was 100% and his Apiezon L column gave good resolution of BHA and BHT. No further work was done on this study at this laboratory. The reason for 50% BHA recovery is unknown.

Recommendation

It is recommended that the revised official first action method for the analysis of BHA and BHT in corn and rice breakfast cereals, as presented in this report, be adopted as official final action.

Acknowledgments

The Associate Referee wishes to thank the following individuals, all of the Food and Drug Administration, for their help in this study: H. A. Walker, Atlanta; T. S. Smith, Baltimore; T. Korbelak, Philadelphia; W. A. Hallam, Boston; A. J. Nero, Chicago; P. L. Imes, Cincinnati; E. J. Campbell, New Orleans; P. Klein, New York; R. H. Johnson, Seattle; D. R. Linsley, Denver; W. M. Ribbron, Detroit; W. G. Schwien, Kansas City; F. G. McNerny, Buffalo; D. Kodama, Los Angeles; R. L. Trauba, Minneapolis; B. L. McGill, Dallas; F. L. Joe, Jr., and J. Rosenstein, Washington, D.C.; and H. W. Gerritz, San Francisco.

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The recommendation of the Associate Referee was approved by the General Referee and by Subcommittee C and was adopted by the Association; their reports will appear in *JAOAC* 53, March 1970.

Collaborative Study on Determination of Extractables from Rubber Materials

By DENNIS E. KORTE[1] (Food and Drug Administration, 240 Hennepin Ave., Minneapolis, Minn. 55401)

Six laboratories studied a method for determining the amounts of water- and hexane-soluble materials from rubber. Four different samples, consisting of small disks of slab rubber, were extracted 7 hr with hexane or water. After the solvent was evaporated, the residues were determined gravimetrically. Satisfactory precision was obtained and it is recommended that the method be adopted as official first action.

A method to determine water- and hexane-soluble materials in rubber was subjected to collaborative study in 1966 (1); Subcommittee C recommended additional collaborative study before adoption of the method (2).

In an attempt to improve the precision of the method, the shape and number of test pieces comprising the test samples were changed. In the previous study, three 2 × 1″ pieces were arranged in a self-supporting triangle on the bottom of the extraction flask. In the present study, 30 or 50 disks are allowed to move freely in the boiling solvent. The use of a large number of disks should minimize errors due to variations in sample thickness, should provide a more uniform surface area, and should increase the amounts of residue recovered resulting in a decrease in relative weighing errors.

Experimental

The test samples consisted of two sets/sample: one set of 30 disks for the hexane extractions and one set of 50 disks for the water extractions. All

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Notes

Gas chromatographic separation of BHA and BHT

The determination of antioxidants in foods can be divided into two parts. One is the removal of the antioxidant from materials in the food which would interfere with the analysis, and the other is the actual analytical determination. The method of preliminary isolation of the antioxidant is obviously dictated by the nature of the food while the final analytical procedure depends on the structure of the antioxidant.

Several colorimetric procedures are available for the analytical determination of BHA (2- and 3-*tert.*-butyl-4-hydroxyanisole) and BHT (3,5-di-*tert.*-butyl-4-hydroxytoluene), but in each case the color measured is unstable and is subject to error by the presence of interfering substances from the food, *e.g.*, reducing sugars, free aldehydes, sodium bisulfite, etc. These procedures are time-consuming and are very dependent on the skill of the analyst in order to obtain accurate results.

The use of gas chromatography for the separation and determination of BHA and BHT overcomes these problems but to date a completely satisfactory column for this work has not been reported. Columns coated with Apiezon L[1], SE-30 silicon rubber gum[2,3] and SE-30 combined with Tween 80[4] are not completely satisfactory because a high operational temperature is required with liquid phase bleeding and consequent background noise and eventual loss of resolving power. In addition the combination column is not easily reproduced[5]. Several liquid phases have been successfully employed with temperature programming[2,3,5], however, they also experience bleeding at the higher temperatures. Since time is required for the column to cool and

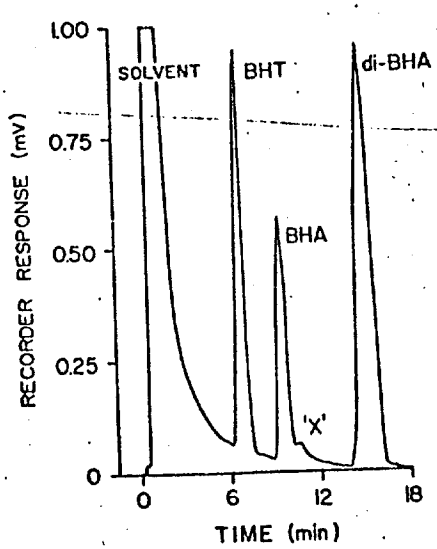

Fig. 1. Gas chromatographic separation of BHT, BHA and di-BHA on Dow Corning QF-1.

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re-equilibrate after an analysis this can be a great inconvenience in a laboratory where a large number of samples have to be run.

In this laboratory a column has been used daily for twelve months without any of the disadvantages outlined above. The liquid phase employed was Dow Corning fluoro-silicone polymer QF-1 (FS-1265). This was coated on HMDS treated 60–80 mesh Chromosorb W at the 20 % level and the stainless steel column measured 5 ft. by $^1/_8$ in. A Wilkins Instrument Company hydrogen flame detector was used in conjunction with a Wilkins Model 500 electrometer and a Honeywell Electronic 15, 0–1 mV recorder. The following operational parameters were employed: column temperature 145°, injection port temperature 160°, carrier gas (nitrogen) 17.0 ml/min, hydrogen 17.0 ml/min, air 900 ml/min.

The separation achieved is shown in Fig. 1. The chromatogram resulted from the injection of 1 μl of petroleum ether (b.p. 35–60°) containing $0.1 \cdot 10^{-3}$ mg BHT, $0.1 \cdot 10^{-3}$ mg BHA, and $0.2 \cdot 10^{-3}$ mg di-BHA (3,5-di-*tert.*-butyl-4-hydroxyanisole). The separation of the three compounds is seen to be complete with very little interference due to tailing of the solvent peak. The analysis of BHA and BHT was completed in 12 min and if the internal standard di-BHA[2] was added an additional 6 min were required. For each compound the peak height was directly proportional to the amount of antioxidant present in the range 0 to $0.5 \cdot 10^{-3}$ mg.

The shoulder "X" following the BHA peak was seen as a distinct peak when $0.5 \cdot 10^{-3}$ mg BHA was gas chromatographed. The areas of the peaks due to BHA and "X" were found to be in the ratio 93:7. Commercial BHA samples contain approximately 94 % of the 3-isomer and the author suspects that the peak denoted by "X" was due to the 2-isomer of BHA.

In the author's laboratory this technique following a preliminary work-up i used to detect antioxidant in the range 0.1 to 10.0 p.p.m. in 100 g samples of potat granules.

American Potato Company, Blackfoot, Idaho (U.S.A.) W. A. B. THOMSON

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Received February 15th, 1965

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OILS, FATS, AND WAXES

Detection of PG, NDGA, BHT, and BHA in Fats and Oils by Thin Layer Chromatography

By SHIRLEY A. SCHEIDT and HARRY W. CONROY (Food and Drug Administration, 1009 Cherry St., Kansas City, Mo. 64106)

PG, NDGA, BHT, and BHA were extracted with acetonitrile from fat or oil, followed by thin layer chromatographic separation and identification. The four antioxidants were distinctly separated from one another by one-dimensional solvent development of the silica gel adsorbent layer. The chromatography required < 1 hour from spotting to visualization with 2,6-dichloroquinone chlorimide. As little as 1 μg of BHT and 0.5 μg of PG, NDGA, and BHA were detected by this method.

Since mixtures of antioxidants are widely used as preservatives in fats and oils, a rapid method is needed for their extraction, separation, and identification. This study was limited to four common antioxidants; propyl gallate (PG), nordihydroguaiaretic acid (NDGA), butylated hydroxytoluene (BHT), and butylated hydroxyanisole (BHA).

A simple extraction of fat or oil to prepare the antioxidants for thin layer chromatography was sought. Lundberg and Halvorson (1) extracted several antioxidants from fat with 80% ethyl alcohol, but BHT was not removed. Schwien and Conroy (2) and Sahasrabudhe (3) extracted BHT from fat with acetonitrile. We found that acetonitrile extracted all four antioxidants from fat or oil dissolved in petroleum ether and that the extract could be used for thin layer chromatography without further purification.

Thin layer chromatography was used for simultaneous separation and identification. Several thin layer methods have been described but none has completely separated PG, NDGA, BHT, and BHA. Seher (4) used two-dimensional thin layer chromatography to separate 12 antioxidants but could not separate PG and NDGA. The two-dimensional method of Sahasrabudhe (3) separated BHT, BHA, PG, and NDGA. However, our laboratory was repeatedly unable to separate PG and NDGA by this method. Conroy and Barnes (5) could not separate PG and NDGA by one-dimensional thin layer chromatography because the antioxidants streaked together on the chromatogram. Salo, Mackinen, and Salminen (6) suggested that PG and NDGA formed a "kritisches Paar" which could not be separated by thin layer chromatography.

Copius-Peereboom (7) used polyamide as an adsorbent, rather than the usual silica gel, and cleanly separated PG and NDGA; BHA, BHT, and NDGA were not separated. Meyer (8) mixed silica gel and kieselguhr together to separate seven antioxidants. Even though the thin layer chromatogram was developed twice in the same solvent, PG and NDGA were only slightly separated.

This paper describes complete separation and identification of PG, NDGA, BHA, and BHT by one-dimensional chromatography on silica gel. Moreover, the persistent problem of streaking has been eliminated.

Samples for this study were prepared by adding 0.003% BHT, BHA, PG, and NDGA to antioxidant-free lard, tallow, and oil.

METHODS

Reagents and Apparatus

(*Note:* All solvents were reagent grade.)

(a) *Mobile solvent.*—Chloroform + methanol + acetic acid, 90:10:2.

(b) *Chromogenic agent, 2.0%.* — Dissolve 2,6-dichloroquinone chlorimide in absolute ethyl alcohol.

(c) *Adsorbent.* — MN-Silica gel G-HR. (Brinkmann Instruments, Inc., Westbury, N.Y.).

(d) *Antioxidants.*—BHT and BHA (Eastman Chemical Products). *n*-Propyl gallate, practical grade (Matheson, Coleman and

Bell). NDGA (nordihydroguaiaretic acid) (Nordigard Corp.). Dissolve 10 mg of each in 10 ml acetone.

(e) *Thin layer applicator.*—To coat plates 0.25 mm thick (Desaga-Brinkmann, Model 3-11, or equivalent).

(f) *Solvent trough.*—About 10″ long and deep enough to hold 0.5 cm solvent.

(g) *Developing tank.*—Glass or metal with tight closure for holding 8″ plate. Line all sides with blotting paper.

Determination

Extraction.—Add 100 ml petroleum ether (30–60°) to 15 g melted fat or oil and transfer to 250 ml separator. Extract with four 25 ml portions of acetonitrile saturated with petroleum ether. Combine acetonitrile extracts in 250 ml beaker and evaporate to 2–4 ml on steam bath with aid of air current. Transfer extract to 5 ml volumetric flask and dilute to volume with acetone. Use this solution for spotting thin layer plates. (Appropriate dilutions can be made for antioxidants present in concentrations higher than 0.003%.)

Preparation of chromatographic plates.—Mix 30 g MN-silica gel G-HR (c) with 60 ml H_2O to form slurry for five 8×8″ plates. Apply layer 0.25 mm thick. Let plates set at room temperature for at least 15 min. Activate plates at 100°C for 30 min. in forced draft oven, cool to room temperature, and store in desiccator.

Preparation of developing tank.—About 30 min. before use, pour mobile solvent (a) into solvent trough to a depth of 0.5 cm. Also, pour mobile solvent (a) on floor of developing tank and place blotting paper in contact with solvent pool.

Spotting and development of thin layer plate.—Spot (microliter pipet) ca 2 μg of each antioxidant (d) and sample 1 cm apart and 1″ from bottom of plate. Develop plate in tank trough, letting solvent front move 10 cm from spotting line. Remove plate and let solvent evaporate at room temperature. (If greater separation between PG and NDGA is desired, return plate to tank and let solvent front move in same direction to 15 cm from spotting line. Remove plate and let solvent evaporate.) Spray with chromogenic agent (b), using a fine mist atomizer. Color development takes ca 5 minutes and may be hastened to ca 30 seconds by carefully exposing the sprayed plate to ammonia vapor. PG appeared as a greenish-gray spot, NDGA gave a red spot, BHT appeared as a yellow spot, and BHA was an orange spot which changed to purple on exposure to ammonia.

Table 1. R_f values of four antioxidants

Antioxidant	Single Solvent Development	Two Solvent Development
NDGA	0.26	0.37
PG	0.32	0.45
BHA	0.74	0.84
BHT	0.90	0.94

Results and Recommendation

Typical R_f values of PG, NDGA, BHT and BHA are given in Table 1. The thin layer chromatogram was developed either to 10 cm, or to 10 cm and then redeveloped to 15 cm.

When silica gel G (Merck) was used, PG formed a brown spot as it was applied to the adsorbent layer and the spot was streaked upon development. However, PG neither formed a brown spot when it was applied to the adsorbent layer nor streaked when it was developed, if the iron in the silica gel was complexed either by prewashing the layer with 2% citric acid or by adding various amounts of citric acid to the slurry. Neither method of treating the adsorbent layer with citric acid aided the separation of PG from NDGA.

When MN-silica gel G-HR (iron content less than 0.001%) was used, PG did not form a brown spot, did not streak, and was completely separated from NDGA. A slight tail on the PG and NDGA spots was eliminated by adding 2% acetic acid to the mobile solvent, as suggested by Bobbitt (9). Less than one hour was required from spotting to visualization. As little as 1 μg of BHT and 0.5 μg of PG, NDGA, and BHA were detected.

This method has made possible a quick extraction, separation, and identification of PG, NDGA, BHT, and BHA with minimal manipulations.

It is recommended that the thin layer

This report of the Associate Referee, H. W. Conroy, was presented at the Seventy-ninth Annual Meeting of the Association of Official Agricultural Chemists, Oct. 11–14, 1965, at Washington, D.C.

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Associate Referee, H. W. Conroy.
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chromatographic method described be studied collaboratively for the quantitative determination of antioxidants.

Acknowledgments

The authors wish to thank David Firestone, William Schwien, and Bob Miller for their helpful suggestions.

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Extraction, Cleanup, and Gas Chromatographic Determination of BHA and BHT in Fats and Oils

By WILLIAM G. SCHWIEN, BOB J. MILLER, and HARRY W. CONROY (Food and Drug Administration, 1009 Cherry St., Kansas City, Mo. 64106)

In preparing samples for analysis of antioxidants in fats and oils, melted fat and/or oil is mixed with Celite, blended with acetonitrile, and the acetonitrile is extracted with mixed ethers. The mixed ethers are evaporated, and the oily residue is incorporated with alumina acid. The alumina mixture is placed in a chromatographic column and eluted with aqueous methanol, and the methanolic eluate is extracted into mixed ethers. The ether extract is clean enough for TLC as well as GLC determinations. Recoveries from tallow, lard, and corn oil spiked at 16.7 and 66.7 ppm with BHA and BHT ranged from 80 to 95% for BHA and 91 to 115% for BHT.

Current methods for butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) in fatty foods include colorimetric determinations from their steam distillates (1, 2) or gas chromatographic procedures by a direct extraction technique (3–5). Colorimetry produces an extract free from fats and oils but requires specialized distilling equipment. Gas chromatography is quite rapid but removes large amounts of fats and oils, along with the antioxidants, which may cause multiple peak interference and column contamination and make any TLC confirmatory testing unworkable. If direct extractions are made on pure fats, residual oil contamination is even more troublesome.

The proposed method involves an acetonitrile extraction and a subsequent cleanup on an alumina acid column. Resulting extracts are clean enough for thin layer chromatography (TLC) as well as gas-liquid chromatography (GLC). The method is rapid and relatively simple, and requires no special equipment for extracting BHA and BHT from fats and oils.

METHOD

Apparatus

(a) *Gas chromatograph.*—Barber-Coleman Model 5000 with an argon ionization detector using a 1 mv strip chart recorder, or equivalent apparatus. A chromatographic column

A Rapid Gas Chromatographic Method for the Determination of BHA and BHT in Vegetable Oils

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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.

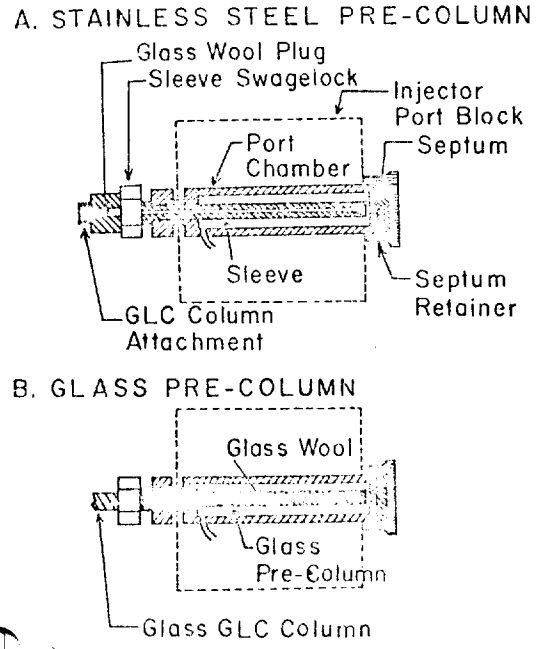

FIG. 1. Diagram of precolumns. Part A: stainless steel precolumn. The stainless steel sleeve is inserted into the port chamber and is secured with Swagelok fittings. Part B: Glass precolumn. The first 5 in. of the GLC column (precolumn) inserts into the port chamber. The column is secured with a Swagelok 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

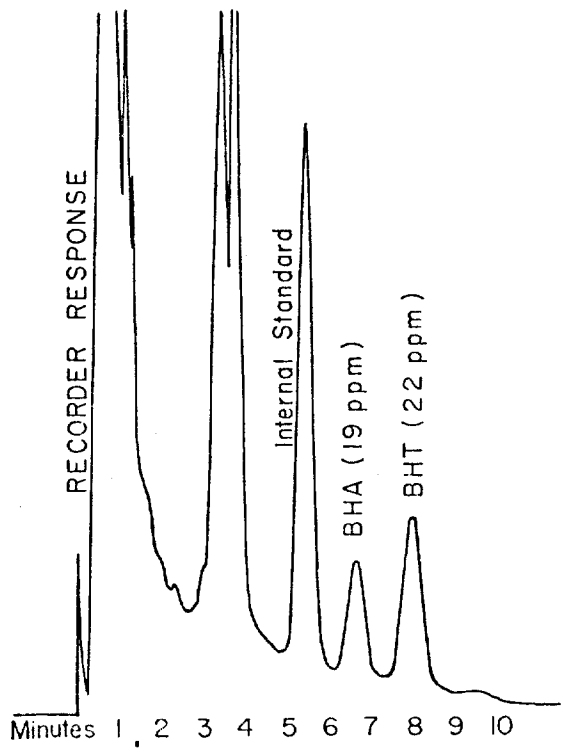

FIG. 2. A 6 ft × 4.5 mm i.d. aluminum column packed with 10% DC 200 on 80–100 mesh Gas Chrom Q, column: 160 C; detector: 250 C; injector port: 250 C; flow-rate: 50 ml/min helium; sensitivity: 4 × 10^{-13} amp. full scale deflection; sample: cottonseed oil fortified with BHA and BHT; sample size injected: 6.0 μl containing 0.100 g oil/ml.

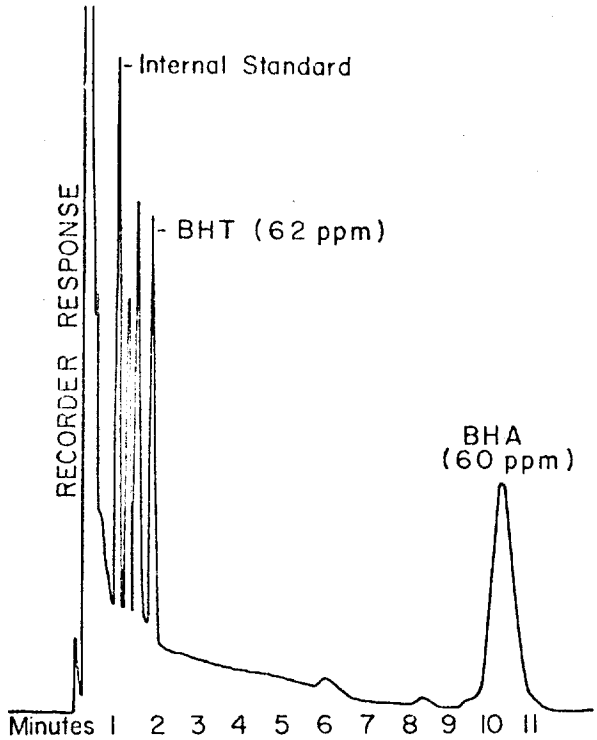

FIG. 3. A 4 ft × 4.5 mm i.d. aluminum column packed with 10% Carbowax 20 M on 80–100 mesh Gas Chrom Q, column: 190 C; remaining conditions same as Figure 2; sample: cottonseed oil fortified with BHA and BHT; sample size injected: 5.0 μl containing 0.100 g oil/ml.

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	101.1
	59.9	104.4	102.6
	99.8	102.1	100.5
Cottonseed	20.0	97.4	98.3
	59.9	97.4	99.2
	99.8	98.0	100.6
Corn	20.0	100.4	98.7
	59.9	100.9	102.8
	99.8	101.3	101.0
Peanut	20.0	103.5	97.8
	59.9	103.4	100.8
	99.8	103.2	98.8
Average (12)		101.1	100.2
Std. Deviation		2.33	1.56
BHT as Antioxidant			
Soybean	20.7	99.2	101.8
	62.2	101.1	101.4
	103.7	100.2	99.4
Cottonseed	20.7	103.8	101.6
	62.2	100.8	102.4
	103.7	99.3	100.0
Corn	20.7	99.1	100.4
	62.2	98.4	98.8
	103.7	100.4	101.8
Peanut	20.7	101.9	98.4
	62.2	102.7	100.6
	103.7	99.9	99.6
Average (12)		100.6	100.5
Std. Deviation		1.53	1.24

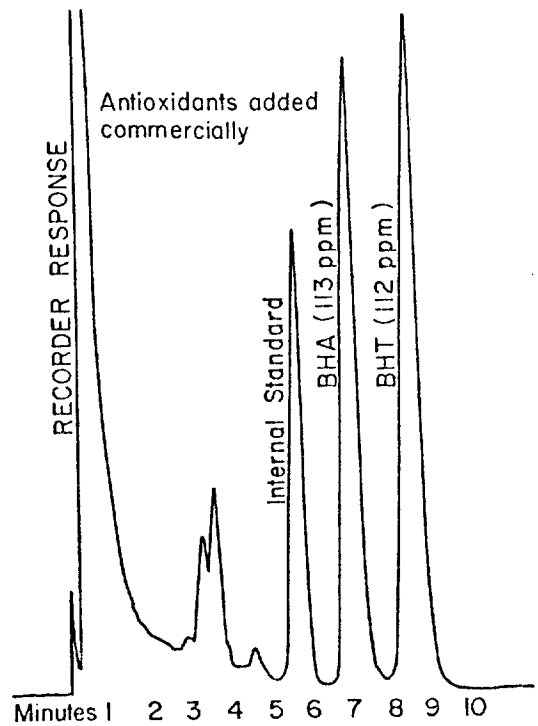

FIG. 4. A 6 ft × 4 mm i.d. glass column packed with 10% DC 200 on 80–100 mesh GAS Chrom Q, column: 15[illegible]; remaining conditions same as Figure 2; sample: special commercially prepared oil; sample size injected: 5.0 μl containing 0.100 g oil/ml.

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 spiked 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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The Determination of Chemical Antioxidants in Fats after Separation by Partition Chromatography*

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Reverse-phase partition chromatography is used to separate butylated hydroxyanisole and the gallate esters from fat. The gallate esters are determined colorimetrically after reaction with ferrous tartrate, and butylated hydroxyanisole after reaction with 2:6-dichloroquinone chloroimide.

A method is described for determining butylated hydroxytoluene in ethanolic solution; it depends on measuring the ultra-violet absorption spectra of strongly alkaline solutions of butylated hydroxytoluene. The method is specific for butylated hydroxytoluene among the permitted antioxidants, but has not yet been successfully applied to its determination in fats.

The quantitative determination of antioxidants in fat involves in the first place their separation from the fat. Methods of separation described in the literature include extraction,[1,2] steam-distillation at elevated temperature[3] and chromatography on moist silica.[4] The application of reverse-phase partition chromatography is described here for this purpose, Silastic 181 (a synthetic rubber) being used as the inert support. The possibility of using both natural and synthetic rubbers for partition chromatography was first suggested by Boldingh.[5] Light petroleum (boiling range 40° to 60° C) is used as the stationary phase and 60 per cent. acetone as the mobile phase. It has not been possible to separate the antioxidants from each other by this technique, but specific methods for determining the gallates and butylated hydroxyanisole (BHA) in the eluate are available.

It was found that the method proposed by Vos, Wessels and Six[2] was suitable for determining the gallates in the aqueous acetone eluates after minor modifications to adapt the procedure to our conditions; it involves reaction with ferrous tartrate and then extraction of the reaction mixture with *iso*amyl alcohol. The purple colour given by octyl or dodecyl gallate is measured in the *iso*amyl alcohol solution, and that derived from propyl gallate can be measured in the aqueous phase.

The determination of BHA has been effected after reaction with 2:6-dichloroquinone chloroimide, a method first introduced by Mahon and Chapman.[1] At first, considerable variations in the colour given by known amounts of BHA were observed. Further, the presence in the test solution of some gallate ester (as might occur in the analysis of commercial fats) caused an increase in the intensity of the colour obtained, although gallates themselves do not react. Investigation of the variables involved showed that the temperature of reaction had a marked effect on the intensity of colour finally reached; more intense colours were obtained at lower reaction temperatures, with an optimum at about 0° C. Optimum values for the pH of the buffer and the solvent composition of the reaction mixture have also been established. When the conditions giving maximum colour development are used for the reaction, no interference is caused by the presence of gallate esters.

A specific method for determining butylated hydroxytoluene (BHT) in ethanolic solution has been devised; it is based on measuring the ultra-violet spectrum of the ionised form of BHT in strongly alkaline solution. Ionisation causes a shift in the absorption maximum from 278 to 306 mμ,[6] and, although this behaviour is characteristic of many phenols (including BHA), it has been found that the alkaline solution of BHT is relatively unstable. The decrease in absorption at the peak wavelength (306 mμ) after storing the solution under controlled conditions gives a quantitative measure of the BHT present. Under the same conditions the ionised form of BHA is relatively stable, and only a small change in absorption at 306 mμ occurs; a correction would need to be applied in analyses of mixtures of BHT and BHA. The ultra-violet spectra of the gallates in alkaline solution have been shown to have no characteristic absorption band. The small absorption at 306 mμ does not change appreciably on storage of the solution, and the presence of gallates should therefore not interfere with this method of determining BHT.

* Presented at the joint meeting of the Society with the Association of Public Analysts on Wednesday, December 3rd, 1958.

The method was devised for determining BHT in the chromatographic eluates [illegible] but accurate quantitative results have not yet been obtained in this application [illegible] is being investigated, and it is hoped that a further communication on the subject [illegible] made in due course.

METHODS

Preparation of Silastic 181—

Advice on the supply of Silastic 181 can be obtained from Midland Silicones [illegible]

Cut the Silastic into small pieces, and pass them through a hammer mill [illegible] or medium sieve and then a second time with a fine sieve. Pass the powdered Silastic [illegible] a sieve, retaining the fraction between 50 and 150 mesh. This is most conveniently [illegible] by immersing the sieve in acetone and brushing the powder through with a [illegible] brush. Allow the solvent to evaporate. Heat the sifted powder to boiling with 5 N [illegible] chloric acid, stir well, decant, and repeat this operation until the washings are free from [illegible] Wash the powder repeatedly with hot distilled water until the washings are free from [illegible] and then extract the powder repeatedly with hot acetone until only a negligible amount of material is removed.

Preparation of columns and separation of antioxidants—

Use analytical-reagent grade light petroleum (boiling range 40° to 60° C) as the stationary phase and 60 per cent. acetone (300 ml of redistilled acetone *plus* 200 ml of water) as mobile phase. Shake the solvents together, and then de-oxygenate by passing oxygen-[illegible] nitrogen through them; use these mutually saturated solvents throughout.

Add 5 ml of light petroleum to 5 g of Silastic, and stir until absorbed. Make [illegible] slurry with 60 per cent. acetone, and transfer the slurry to a chromatographic tube. Run [illegible] excess of mobile solvent until the Silastic just remains covered. It has been found convenient to use a tube about 35 cm long, the upper 20 to 25 cm being wider (2·5 cm diameter) [illegible] the lower portion (1 cm diameter). The tube is water-jacketed and maintained at 21 [illegible]

Weigh 3 to 4 g of fat, and dissolve it in the minimum amount of light petroleum. [illegible] 1 g of dry Silastic for each gram of fat and each millilitre of solvent used. Stir well, [illegible] into a slurry with the mobile solvent, and transfer to the column. Use 2 to 3 ml of [illegible] petroleum to rinse the beaker, and absorb in 3 g of Silastic. Transfer this to the column with 60 per cent. acetone.

Wash the column with 60 per cent. acetone at the rate of about 150 ml per hour. Collect 200 ml *plus* 100 ml of eluate, and analyse separate portions of these eluates for BHA and [illegible] gallate esters. When the sample contains not more than about 300 μg of these antioxidants they will normally be present in the first 200 ml of eluate, and the collection of two separate fractions avoids the unnecessary dilution that would result from collecting one 300-ml fraction.

Determination of butylated hydroxyanisole

Reagents—

Ethanol, 95 per cent.—Analytical-reagent grade.

2:6-Dichloroquinone chloroimide reagent solution—A 0·02 per cent. solution of 2:6-dichloro-*p*-benzoquinone-4-chloroimine in 95 per cent. ethanol.

Buffer solution, pH 9·8—Mix two volumes of 0·05 *M* sodium carbonate with one volume of 0·05 *M* sodium tetraborate, both solutions having been prepared from analytical-reagent grade salts.

Procedure—

Take 10-ml aliquots from each fraction in 25-ml calibrated flasks. Add 6 ml of 95 per cent. ethanol to each, and cool to 0° C. Add 2 ml of 2:6-dichloroquinone chloroimide reagent solution previously cooled to 0° C. Mix, and add 3 ml of buffer solution previously cooled to 0° C. Mix, and allow the colour to develop for 1 hour at 0° C; then set the solution aside (for about 30 minutes) until it has reached room temperature, and make up to the [illegible] with 60 per cent. acetone.

Measure the optical density against 60 per cent. acetone in 4-cm cells at 610 mμ with a Unicam SP600 spectrophotometer. Calculate the BHA content of the fractions by [illegible] a standard graph prepared by measuring under the same conditions the optical density of known amounts of BHA (up to 40 μg).

Determination of gallates

Reagents—

Ferrous tartrate reagent solution—Dissolve 0·1 g of ferrous sulphate and 0·5 g of Rochelle salt in 100 ml of distilled water. Before use, add to 10 ml of this solution 2 ml of a 2 per cent. solution of sulphur dioxide in water, and set aside for 10 minutes.

Sodium acetate solution—A 1 per cent. solution of sodium acetate (analytical-reagent grade) in water.

Amyl alcohol—"*iso*Amyl Alcohol for Milk Testing."

Extraction solvent—Equal volumes of amyl alcohol and light petroleum (boiling range 40° to 60° C).

Acetone—Redistilled.

Buffer solution, pH 4·6—Mix four volumes of 0·1 *M* potassium hydrogen phthalate with one volume of 0·1 *M* sodium hydroxide, both solutions having been prepared from analytical-reagent grade salts.

Procedure—

Measure 60 to 80 ml of eluate into a 250-ml separating funnel. Add 1·2 ml of the freshly "reduced" ferrous tartrate reagent solution. Add a volume of sodium acetate solution equal to the volume of eluate taken. After 10 minutes extract the solution first with 20 ml and then with 15 ml of the extraction solvent. Dilute the combined extracts to 50 ml with amyl alcohol, and determine the higher gallates by measuring the optical density in a 4-cm cell at 532 mμ with a Unicam SP600 spectrophotometer.

Determine propyl gallate by measuring the optical density of the aqueous phase at 532 mμ after diluting to 200 ml with acetone. (In view of the weak colours obtained, we have preferred to make this measurement in a 20-cm cell on a Spekker absorptiometer with a Chance OG1 filter.)

Calculate the gallate content of the solution by the use of standard graphs prepared by treating known amounts of each of the gallate esters (up to a total of 200 μg) by the method described above.

A qualitative test for differentiating between octyl and lauryl gallates has been described by Vos, Wessels and Six,[2] but it was found to be useless when applied to the eluates obtained by the chromatographic method.

The test described below has, however, been shown to distinguish between the two higher gallates.

Carry out the ferrous tartrate reaction on about 20 ml of the eluate, as described above, but use the phthalate buffer solution in place of the sodium acetate solution, and extract with 5 ml of the extraction solvent. A blue colour is given in the upper layer by lauryl gallate, whereas no colour is obtained in either layer with octyl gallate. It is hoped that this test may be developed into a quantitative method specific for lauryl gallate; however, for many purposes a qualitative differentiation between the two gallates is all that is necessary.

Determination of butylated hydroxytoluene

Reagents—

Ethanol, 80 per cent.—Dilute 80 ml of ethanol (analytical-reagent grade, 99 to 100 per cent., optical transmission at least 95 per cent. at 270 mμ) with 20 ml of water distilled in all-glass apparatus. De-oxygenate before use by bubbling oxygen-free nitrogen through the solvent.

Potassium hydroxide solution—Place 47 ml of water distilled in all-glass apparatus in a 200-ml flask. Pass a slow stream of oxygen-free nitrogen through the water for about 10 minutes. Add 53 g of analytical-reagent grade potassium hydroxide, and continue the passage of nitrogen until complete solution is effected. Insert the stopper in the flask, and set aside until cool. This alkali may be prepared in larger quantities and stored, preferably in a polythene container.

Procedure—

Measure 10 ml of BHT solution in 80 per cent. ethanol (containing 10 to 100 μg of BHT) into a 15-ml glass-stoppered calibrated flask, and cool to between 0° and 5° C. Add by pipette (preferably of the syringe type) 4 ml of potassium hydroxide solution, mixing well during addition to prevent formation of a separate layer of alkali. Dilute to the mark with 80 per

cent. ethanol. Exactly 5 minutes after beginning to add the alkali, measure the optical density at 306 mμ in a 4-cm cell against distilled water. Return the solution to the flask and place the flask in a thermostatically controlled bath at 31° C. After holding for 2 hours at 31° C again measure the optical density at 306 mμ.

Treat 10 ml of pure solvent by the procedure described above. Only a small change in the optical density of the blank solution (less than 0·010) should occur after holding at 31° C. Subtract the corresponding readings from those obtained for the BHT solution to give the net optical densities.

The alkalinity of the final solution should be checked by titration and should lie outside the limits 3·0 to 3·2 N.

The decrease in the net optical density after holding at 31° C has been shown to be a measure of the concentration of BHT, a drop of 0·33 being obtained when 160 μg of antioxidant are present in the 10 ml of solution taken for the determination.

TABLE I

RECOVERY OF MIXTURES OF ANTIOXIDANTS

	Butylated hydroxyanisole			Octyl gallate			Propyl gallate		
	Added, μg per g	Found, μg per g	Error, μg per g	Added, μg per g	Found, μg per g	Error, μg per g	Added, μg per g	Found, μg per g	Error, μg per g
Groundnut oil ..	—	—	—	39	43	+4	64	60	−4
	—	—	—	66	77	+11	38	40	+2
Palm-kernel oil..	189	193	+4	42	38	−4	—	—	—
	134	133	−1	10	11	+1	—	—	—
	97	96	−1	99	103	+4	—	—	—
	95	98	+3	45	50	+5	—	—	—
	199	199	0	—	—	—	61	64	+3
	202	209	+7	—	—	—	101	101	0

RESULTS AND DISCUSSION OF THE METHOD

The results obtained for analyses in which known amounts of BHA or of one of the gallate esters were added to fats are shown in Fig. 1. The fats used were palm-kernel oil, groundnut

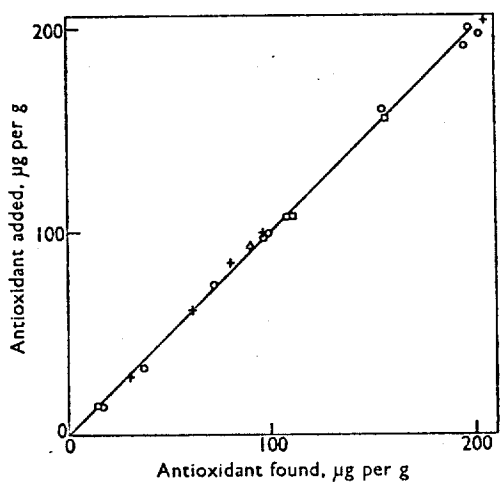

Fig. 1. Recovery of antioxidants added to fats: ○, butylated hydroxyanisole; □, propyl gallate; +, octyl gallate; △, lauryl gallate

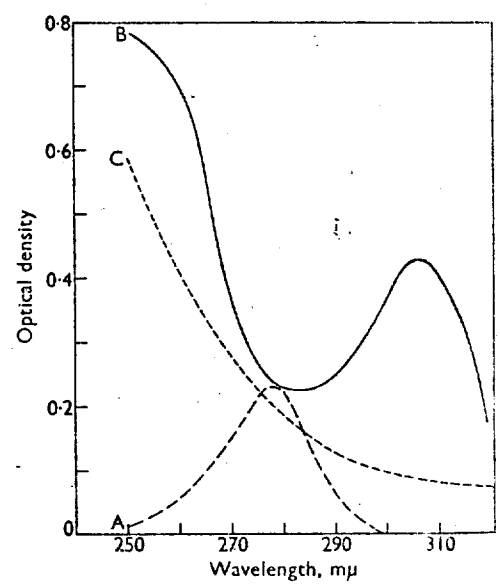

Fig. 2. Ultra-violet absorption spectra of butylated hydroxytoluene: curve A, in 80 per cent ethanol; curve B, 5 minutes after addition of alkali; curve C, after standing for 2 hours at 31° C

oil and suet fat, and in addition to these results the method has been applied successfully to the determination of octyl gallate in orange oil (995 μg per g added; 980 μg per g recovered). Results obtained for analyses in which a mixture of two permitted antioxidants has been added to fats are shown in Table I. A satisfactory degree of accuracy has been attained in all these determinations.

The ultra-violet spectra for BHT, BHA and octyl gallate (100 μg in 15 ml of solvent) are shown in Figs. 2, 3 and 4, together with the corresponding spectra after the addition of alkali and after storing the alkaline solutions at 31° C. For the treatment with alkali, 100 μg of antioxidant were also taken, and the procedure described for the determination of BHT was followed in each instance.

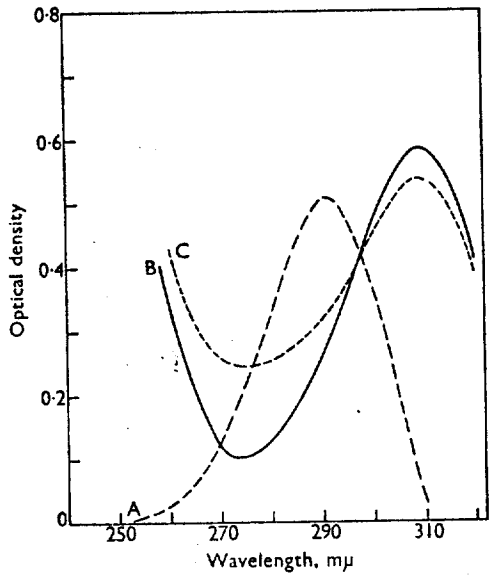

Fig. 3. Ultra-violet absorption spectra of butylated hydroxyanisole: curve A, in 80 per cent. ethanol; curve B, 5 minutes after addition of alkali; curve C, after standing for 2 hours at 31° C

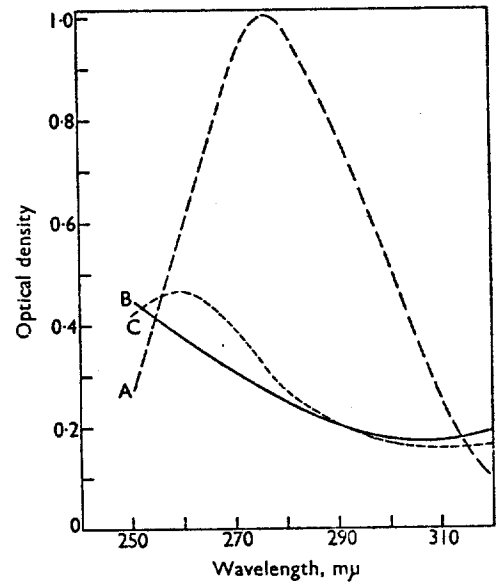

Fig. 4. Ultra-violet absorption spectra of octyl gallate; curve A, in 80 per cent. ethanol; curve B 5 minutes after addition of alkali; curve C, after standing for 2 hours at 31° C

The procedure adopted is empirical, and a similar effect can be obtained by setting aside at lower temperatures for a longer time. The conditions described are conveniently available in our laboratory, and it is not suggested that an alternative standardised method could not be devised.

It can be seen from Figs. 2 and 3 that the absorption maximum at 306 mμ for BHT disappears completely after storage for 2 hours at 31° C, whereas only a small change occurs in the absorption spectrum of BHA (maximum at 309 mμ) under the same conditions. The change in the net optical density of the alkaline solution of BHA at 306 mμ is about 14 per cent. of that exhibited by a solution of BHT at the same concentration, and a correction (calculated from the pre-determined amount of BHA present) will be necessary if it is eventually possible to apply these observations to the determination of BHT in fats.

We thank Dr. E. B. Hughes for his interest and encouragement and the Directors of J. Lyons & Co. Ltd. for permission to publish.

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Discussion

Mr. J. Markland congratulated the authors on their interesting paper and said that he was that he could speak for Public Analysts and say that they were indebted to the Food Industry for tributing valuable material on a problem common to local authorities and manufacturers.

He had been working on this problem and had used a similar method of partition chromatography but different materials. This work started with the qualitative detection of antioxidants by chromatography on paper impregnated with liquid paraffin (Markland, *J. A.P.A.*, followed by attempts at separation on a silica column with dilute alcohol as the stationary light petroleum as mobile phase. The gallates were completely removed, but butylated hydroxy passed through. This confirmed the authors' experience. However, if traces of iron were removed the silica by washing with hydrochloric acid or if an acidified alcohol was used as the stationary some separation was obtained. The retention of the gallates might have been due to a combination a slightly alkaline silica gel and traces of iron.

This method of separation was abandoned in favour of polythene columns treated with liquid as stationary phase. Dilute alcohol was used as mobile phase. By increasing the concentration of used, the various antioxidants could be separated. This work was not yet completed.

The separation of antioxidants, except butylated hydroxytoluene, from fat had been extraction of a solution of the fat in light petroleum with 80 per cent. alcohol. Three extractions quantitative recovery of the gallates.

Mr. C. B. Stuffins said that, after a considerable amount of investigation, it had been found butylated hydroxytoluene was certainly the most difficult to extract from fats. It had been that butylated hydroxytoluene formed a complex with the fat, and this might be a possible reason the difficulty.

Dr. E. C. Wood asked where Silastic 181 could be obtained; could it be purchased ready prepared use in the authors' analytical method, and what led the authors to try this substance out of all the others that might have been considered.

Mr. Berger replied that Silastic was one of the materials suggested for partition chromatography by Boldingh (*Rec. Trav. Chim. Pays-Bas*, 1950, **69**, 247), although he gave no actual example of its Mealorub (a powdered natural rubber used by Boldingh) was found to discolour and oxidise somewhat, and was not considered suitable for their work. Silicone rubbers were very inert and almost ideal reverse-phase chromatography. After consultation with Midland Silicones they selected Silastic being the least likely to contain interfering substances.

Mr. K. A. Hyde asked if the study of antioxidants in fats in relation to induction period threw light on the potential life of antioxidants in fats.

Mr. Berger said that the work described had not dealt with the effectiveness or mode of action the antioxidants, but it was considered that their methods would be useful in investigating these problems.

Mr. H. S. Young enquired if it could be assumed that no oxidation of the fat would take place as there was even a trace of antioxidant present.

Mr. Berger replied that this was a matter that had been extensively investigated (J. H. Mahon and R. A. Chapman, *J. Amer. Oil Chem. Soc.*, 1953, **30**, 34 and 1954, **31**, 108), and it appeared that oxidative rancidity occurred before the antioxidant was completely destroyed.

Mr. G. S. Ingram said that the authors stated that a reaction period of one hour was allowed for the reaction between butylated hydroxyanisole and Gibbs' reagent. Was this quite necessary for both alcohol and acetone.

Mr. Berger said that the conditions specified for the Gibbs' reaction were found to give maximum colour development when butylated hydroxyanisole was dissolved in the eluting solvent used in the chromatographic separation. It was quite possible that other optimum conditions would obtain when a different solvent was used.

Dr. W. E. Elstow said he had found that, in aqueous solutions at least, the rate of colour development from butylated hydroxyanisole and Gibbs' reagent depended on the pH and that, although some time had to be allowed for the colour to develop at pH 9·3, the development reached the same maximum almost immediately at pH 10·4.

Dr. J. H. Hamence asked whether any attempts had been made to separate the individual antioxidants by paper chromatography after column separation. From his experience in this matter it seemed to him that the various antioxidants were substances that should lend themselves to separation by paper chromatography.

Mr. Berger replied that they had aimed at an accurate determination of permitted antioxidants and that they had not made any attempt to analyse their eluates by paper chromatography. Complete separation of the antioxidants by paper chromatography had not yet been effected, but it would prove a useful method of identification in due course.

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A Direct Spectrophotometric Determination of Butylated Hydroxyanisole in Lard and in Hardened Lard

P. V. HANSEN,[1] F. L. KAUFFMAN, and L. H. WIEDERMANN, Research Laboratories, Swift and Company, Chicago, Illinois

THE USES and advantages of antioxidants in edible fats and oils have been adequately described in the literature (5, 6, 10, 11); however the development of methods for the determination of these antioxidants has lagged considerably behind the application of these stabilizers in fat products. With a few exceptions (1, 7, 10, 13) all of the published methods for the estimation of edible antioxidants in fats and oils depend upon the formation of a color complex which can be measured in the region of the visible spectrum (1, 2, 3, 9, 12). These colorimetric methods are suited for systems containing one or more antioxidants when they are present in appreciable quantities.

Two areas of particular deficiency encountered in the above methods are that they do not allow rapid and convenient procedures nor are they effective for small quantities of antioxidant(s), *i.e.*, a few parts per million (p.p.m.). Since butylated hydroxyanisole [BHA; 2-(and 3-)*tert*-butyl-4-hydroxyanisole] and butylated hydroxytoluene (BHT; 3,5-di-*tert*-butyl-4-hydroxytoluene) are of particular interest in fat technology, it is necessary to have available a convenient method for their quantitative determination over a practical range of concentrations having 100 p.p.m. as its higher limit.[2]

Hall and Clark (7) have reported the detection of BHA and other antioxidants by infrared analysis, and this approach may offer definite possibilities for the development of a quantitative method of analysis. Whetsel *et al.* (13) have developed a direct spectrophotometric method for the determination of BHA and propyl gallate in commercial antioxidant preparations. Their method takes advantage of the *iso* absorptive points of the absorption curves of the two substances; however its practical limitations in the low range of antioxidant concentrations have not been explored, nor does it provide either the presence or separation of oleaginous materials. In a recent publication (10) a method for analyzing butylated hydroxytoluene in lard and shortenings was described. This method utilizes chromatographic separation for the isolation of the antioxidant in the eluate, which is then analyzed for BHT by using its ultraviolet absorption curve between 240 and 320 $m\mu$. This is a satisfactory method for the analysis of BHT (BHA being noninterfering); however its applicability over an extended range of antioxidant concentrations has not been reported.

The present paper describes a method for determining butylated hydroxyanisole in lard and in hardened (iodine value less than 10) lard by means of a direct spectrophotometric technique and allows the estimation of the antioxidant when it is present in amounts as low as a few parts per million. This method is based on the strong absorbability of BHA at 290 $m\mu$ because of the hydroxyl group in the aromatic ring. The quantity of antioxidant present is determined from a standard curve after its extraction from the fat sample with methanol. It should be noted that such other antioxidants as BHT and tocopherols interfere with this determination, and this method cannot be successfully applied to BHA in their presence.

Experimental

Materials. Food-grade butylated hydroxyanisole was used and was obtained from Eastman Chemical Products Inc., Kingsport, Tenn. The fats used in the sample preparations were a) deodorized, prime steamed lard (66.5 iodine value) and b) deodorized, commercially hardened lard (6.5 iodine value). The solvents used, chloroform and methanol, were ACS grade. Spectrophotometric analyses were made with a Beckman DU spectropohotometer, using matched 1-cm. silica cells and a hydrogen lamp.

Method. Weigh 25.0 g. of hardened lard into a 250-ml. glass-stoppered flask, melt on a steam bath, and add 10 ml. of chloroform and 100 ml. of methanol. Heat to just below the boiling point of the solvent. Shake vigorously for 1 min. while the contents of the flask are still molten. Then chill in an ice bath to precipitate most of the dissolved fat, filter through No. 1 Whatman filter paper, and set aside the methanol layer. Remelt the remaining fat, add 5 ml. of chloroform and 50 ml. of methanol, and extract as before. Repeat this procedure a third time. Evaporate the combined extracts on a steam bath to a volume of approximately 10 ml., chill thoroughly in an ice bath, and filter through No. 1 Whatman filter paper into a 25-ml. volumetric flask. Wash filter with previously chilled methanol and, after attaining room temperature, dilute the contents of the flask to volume with methanol.

The extraction of BHA from lard was performed in much the same manner as described above for hardened lard. In place of a glass-stoppered flask the extraction was carried out in a separatory funnel at a temperature just below the boiling point of the solvent mixture. After cooling to room temperature, the methanol layer is decanted and filtered through No. 1 Whatman filter paper. This procedure is repeated three times, and the combined extracts are handled as described above.

Spectrophotometric readings were made by filling a 1-cm. silica cell with the solution and measuring its optical density at 270, 290, and 310 $m\mu$ with the

[1] Present address: Dana College, Blair, Nebr.
[2] The maximum level (0.01%) allowed by the Meat Inspection Bureau.

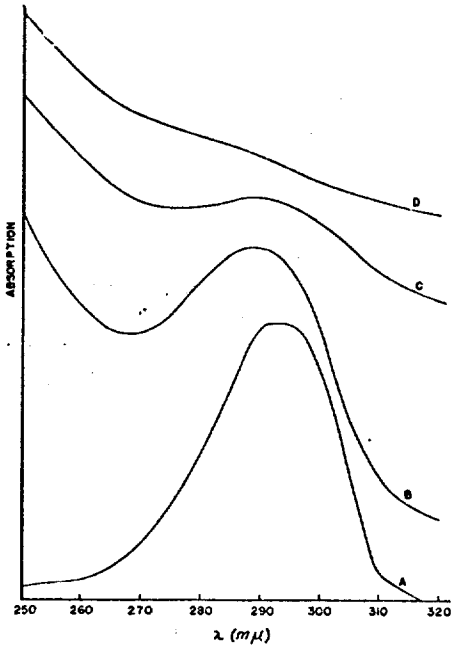

FIG. 1. Ultraviolet absorption curves of methanol solutions (curves are displaced on the vertical axis): A, containing 50 p.p.m. BHA; B, from the extraction of hardened lard containing 50 p.p.m. BHA; C, like B, containing 10 p.p.m. BHA; D, like B, containing no added BHA.

instrument adjusted to read 0.00 optical density for the solvent.

The corrected optical density (to account for dissolved fat) is calculated from the formula:

$$d_{1(290)} = d_{290} - \left(\frac{d_{270} - d_{310}}{2} \right)$$

where $d_{1(290)}$ is corrected optical density at 290 $m\mu$
d_{290} is actual optical density at 290 $m\mu$
d_{270} is actual optical density at 270 $m\mu$
d_{310} is actual optical density at 310 $m\mu$

The parts per million of BHA in the sample are read from a standard curve made by plotting corrected optical density [$d_{1(290)}$] *versus* p.p.m. BHA of known samples.

Results and Discussion

The effect of dissolved fat on the ultraviolet curve for an alcoholic solution of BHA is shown in Figure 1, as are the effects of fat absorption with changing BHA concentrations. The significant changes observed are the rounding-off of the characteristic peak for BHA at 290 $m\mu$. These changes can however be compensated for and will not adversely affect the determination of BHA in these solutions.

To correct for dissolved fat a straight line is drawn through the points at 270 and 310 $m\mu$ of an ultraviolet absorption curve for the solution made between 250 and 320 $m\mu$, as shown in Figure 2. These points were chosen because the straight line, ab, is then tangent to the characteristic minimum and the nearly flat portion of the curve.[3] Alcoholic solutions containing dissolved fat with varying quantities of BHA produce curves of generally the same form with the peak of the curve at approximately 290 $m\mu$ being shifted upward with an increase in the BHA content. The effect of small amounts of dissolved fat is to shift the whole curve upward. This does not affect the distance from the straight line, ab, to the peak or point of the curve at 290 $m\mu$, line cd, but only increases the distance from the abscissa to this peak. This then gives a basis for the measurement of the BHA concentration in a solution containing dissolved fat. Rather than plot each set of points and measure the distance cd, it was found satisfactory to subtract the mean of the readings at 270 and 310 $m\mu$, point *d*, from the reading at 290 $m\mu$, point c, thus giving the optical density resulting from only the BHA in the solution under investigation.

A plot of the corrected optical density values from the extraction of hardened lard samples against the known BHA content of these samples gives a linear relationship (curve A in Figure 3). The small correction because of the slight absorption at 290 $m\mu$ of solutions from the extraction of samples containing no BHA is inherent in the $d_{1(290)}$ *vs.* $m\mu$ plot in Figure 3.

The validity of this curve was further checked by the analysis of samples containing known levels of BHA, particularly in the lower range of concentrations. These data are contained in Table I and generally show an accuracy of plus-or-minus 2 p.p.m. for BHA concentrations below 50 p.p.m. This allows analysis of samples containing approximately 2 p.p.m. of BHA.

Curve B in Figure 3 also shows a straight line rela-

TABLE I

Recovery of BHA from Known Samples of Hardened Lard

BHA added (p.p.m.)	BHA recovered (p.p.m.)						
	Trial					Avg.	Deviation
	I	II	III	IV	V		
3	3.5	3.2	2.85	2.7	3.15	3.07	±0.4
6	5.75	5.9	5.8	6.0	5.8	5.85	±0.2
9	9.1	8.7	9.2	9.2	9.25	9.09	±0.3
25	24.2	....	....	....	....	....	....
50	51.0	....	....	....	....	....	....
100	95.3	....	....	....	....	....	....

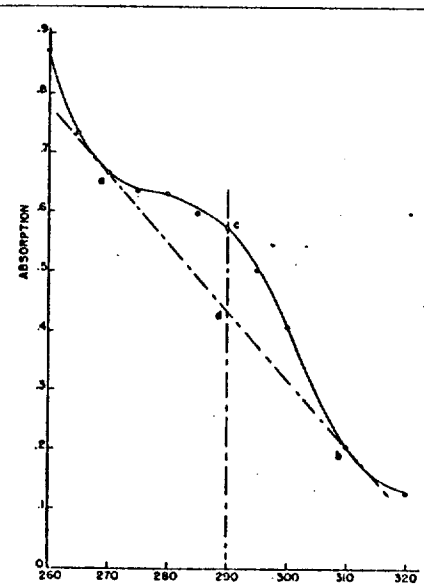

FIG. 2. Spectrophotometric curve of alcoholic solution from the extraction of hardened lard containing 10 p.p.m. BHA. Distance cd is a direct measure of absorption because of BHA and is independent of any dissolved fat.

[3] The minimum at 270 $m\mu$ is observed for curves of solutions containing low BHA concentrations, and while this point shifts slightly at higher BHA levels (to 265 $m\mu$ at 100 p.p.m.), the use of its optical density at 270 $m\mu$ was found to give consistent results throughout the tested range of concentration.

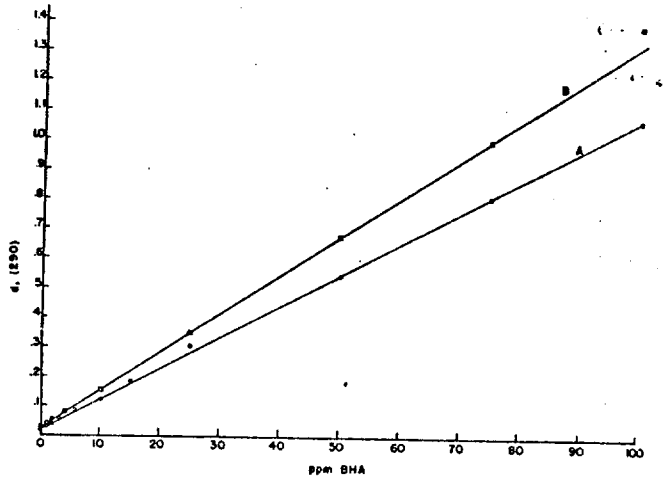

FIG. 3. Corrected optical density *versus* p.p.m. BHA in known samples: A, curve for hardened lard samples; B, curve for lard samples.

tionship when this method is applied to the determination of BHA in lard samples. With fats much softer than lard the accuracy of this method falls off rapidly on account of their increased solubility in the solvent. When measures are taken further to decrease their solubility, the solubility of BHA is also reduced but not to a reproducible level. It is therefore a necessary restriction of this method that the fat samples under investigation not be softer than lard or rather that they do not contain more lower-melting, more-soluble triglycerides.

This method was used to determine how effectively BHA is removed from lard or hardened lard during deodorization. In the deodorization process steam is bubbled through the fat, which has been heated to a temperature of 220°C. at 1-mm. pressure for 4 hr. Under these conditions the steam distillable BHA should be completely removed from the fat. Thirty p.p.m. of BHA were added to hardened lard both before and after deodorization, and samples of each were then analyzed by the present method. Table II compares these results with a control as well as the analysis of these samples by the 2,2′-bipyridine-ferric chloride method. The colorimetric procedure for analysis gives low results, and while it does indicate the complete removal of BHA during deodorization, the results cannot be considered reliable for low concentrations of BHA. The use of borax-2,6-dichloroquinonechlorimide (9) is under optimum conditions capable of the qualitative detection of BHA. However this method is of little practical value to the processor who has to restabilize a deodorized stabilized lard (11).

TABLE II

BHA Content of Hardened Lard Before and After Deodorization

	BHA recovered (p.p.m.)	
	Present method	Colorimetric method (1, 9)
1. Control (no BHA added)	0	0
2. 30 p.p.m. BHA added to control	29.3	20
3. Sample 2 after deodorization	0	0
4. 30 p.p.m. added to Sample 3	29.6	20

It was observed that, on re-examination of the hardened lard-BHA test samples after 40 and 100 days of storage at room temperature, there was a decrease in the antioxidant level. After 40 days there were 68 p.p.m. and after 100 days 57 p.p.m. for the 100-p.p.m. BHA sample. This decrease was found to be proportional at all BHA concentrations at each time interval, thus exhibiting a continued linear relationship. We can attribute this to the oxidation of BHA in its role as an antioxidant with the loss of its characteristic phenol-grouping. This loss through oxidation is not thoroughly understood, but it is thought that the isomeric phenoxy radicals will rearrange to give a semiquinone structure (8) or, as in the case of the oxidation of butylated hydroxytoluene (4), undergo some degree of dimerization. Either path would result in the observed decrease of BHA.

Summary

A direct spectrophotometric method for the determination of butylated hydroxyanisole has been presented. This procedure allows good precision in the analysis of lard and hardened lard containing from 0 to 100 p.p.m. of BHA and is more reliable than existing colorimetric techniques, especially in the lower range of antioxidant concentrations.

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Analysis of Surfactant Mixtures. I.

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MOST SCHEMES for characterizing or analyzing surfactants rely on specific reactions of the hydrophilic part of the molecule (*e.g.*, sulfate, sulfonate, or polyethoxyethanol groups). Much additional useful information can be obtained by also identifying the hydrophobic raw material used in the manufacture of the surfactant. Identification of the hydrophobic portion of the molecule is very difficult on the intact surfactant and can be accomplished much more easily if it can be decomposed into a

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Fluorimetric Determination of Butylated Hydroxy Anisole in Food Products and Packaging Material

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A fluorimetric method for the determination of butylated hydroxy anisole in lard, ready-to-eat cereals, and waxed packaging liners is described. Solutions of lard samples are analyzed directly by a standard addition method that corrects for the interference due to propyl gallate or butylated hydroxy toluene. Samples of cereals and packaging materials require prior separation of butylated hydroxy anisole by combination of extraction and thin-layer chromatography. In all procedures the native fluorescence of butylated hydroxy anisole is measured; thus, the problems encountered with colorimetric methods are avoided.

BHA (2- and 3-*tert*-butyl-4-hydroxyanisole) is widely used in many food products and waxed package liners as an antioxidant to preserve freshness. It is used alone, or together with other antioxidants such as BHT (3,5-di-*tert*-butyl-4-hydroxytoluene) and PG (*n*-propyl gallate). Mixtures of the antioxidants are more effective in preserving freshness since no one compound is equally effective in all food products under all conditions. Because of government regulations and the presence of other antioxidants, an analytical method for the determination of BHA in food products must be sensitive in the parts per million range, as well as being specific, rapid, and accurate.

Sahasrabudhe (1964) has successfully utilized thin-layer chromatography to determine quantitatively BHA, BHT, PG, and NDGA in lard. McCaulley *et al.* (1967) have developed a vacuum sublimation method for isolating the antioxidants BHA, BHT, Ionox-100, PG, THBP, and TDPA from lard and quantitatively determining the antioxidants by gas chromatography. Johnson (1967) took advantage of the open *ortho* position in the BHA molecule to form a colored nitroso derivative, and spectrophotometrically determined BHA in vegetable oils. Recently Takahashi (1967) developed a gas chromatographic method using a hydrogen flame ionization detection system to determine BHA and BHT in ready-to-eat corn and rice cereals. In a later publication, Takahashi (1968) gave the results of a collaborative study for the two phenolic antioxidants. Schwecke and Nelson (1964) also used gas chromatography with a flame ionization detector to determine BHA and BHT in ready-to-eat cereals, potato products, and waxed paper.

This paper describes a new approach for the quantitative determination of BHA in lard, ready-to-eat cereals, and waxed package liners. The native fluorescence of BHA is used to determine quantitatively BHA directly in lard either alone or in the presence of BHT and PG. BHA in cereal and in packaging materials is determined by the fluorescence emission of BHA after separation of the antioxidant.

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EXPERIMENTAL

Apparatus and Equipment. All fluorescence and phosphorescence measurements were obtained with an Aminco-Bowman spectrophotofluorometer equipped with a phosphoroscope accessory. The source for the excitation monochromator was a Xenon lamp (American Instrument Co., Catalog No. 416-992). The detector for the emission monochromator was a 1P21 phototube or 1P28 phototube. The latter tube provided greater sensitivity.

The thin-layer chromatographic equipment consisted of a rectangular Kensco developing tank, 10 × 20 cm glass plates, plastic template to apply accurately and locate spots, and a "moving plate" apparatus to coat the glass plates. A Hamilton 50-μl or 10-μl syringe was used to spot samples on thin-layer plates.

Reagents. ABSOLUTE ETHANOL. The ethanol was distilled once using a distilling column similar to the one described by Winefordner and Tin (1964).

LIGROINE (bp 63–75° C). Eastman practical grade treated the same as ethanol.

N,2,6-TRICHLORO-P-BENZOQUINONEIMINE. A 2.0% solution of the Eastman compound was made up in absolute ethanol.

BHA, BHT, AND PG. These food grade antioxidants were used without further purification and were obtained from Eastman Chemical Products, Inc., Kingsport, Tenn., and Universal Oil Products Co., East Rutherford, N.J.

QUININE SULFATE. A stock solution of 0.8 μg per ml of quinine sulfate in 0.1N H_2SO_4 was used daily to check the sensitivity of the spectrophotofluorometer for lard analyses. The excitation monochromator was set at 250 nm and the emission monochromator was set at 450 nm. The sensitivity was then adjusted to the same value as previously obtained using a 1P21 phototube.

BHA. A stock solution of 2.0 μg per ml of BHA in ethanol was used daily to check the sensitivity of the spectrophoto-

fluorometer for cereal and packaging analyses. The excitation monochromator was set at 290 nm and the emission monochromator was set at 327 nm. The sensitivity was then adjusted to the same value as previously obtained using a 1P28 phototube.

DETERMINATION OF BHA IN LARD

The cell used to measure the fluorescence intensity of the antioxidants must be cleaned and completely dry before each reading. Also, if PG is present, its concentration must be previously determined. This can be easily accomplished by using the method of Latz and Hurtubise (1969) for determining PG directly in lard without interference from either BHA or BHT.

Separate standard solutions of BHA and PG were prepared containing 10.0 μg per ml of food grade antioxidant in 50% ethanol-ligroine. Between 2.400–2.600 g of lard were weighed in a 100-ml beaker, melted, and dissolved in approximately 50 ml of 50% ethanol-ligroine. The sample was quantitatively transferred to a 100-ml volumetric flask and diluted to volume. Aliquots of 10 ml of lard solution were placed in seven 25-ml volumetric flasks. Samples of 5.0, 2.0, and 1.0 ml of the standard BHA solution were added to three of the volumetric flasks. Then samples of 5.0, 2.0, and 1.0 ml of the standard PG solution were added to three of the remaining four volumetric flasks. All seven flasks were diluted to 25 ml with 50% ethanol-ligroine. The spectrophotofluorometer was adjusted with a standard solution of quinine sulfate using slit arrangement five. The relative fluorescence intensities of the seven lard solutions were measured using a 1P21 phototube with the excitation monochromator at 292 nm and the emission monochromator at 342 nm. The relative fluorescence intensity obtained from the blank lard solution was subtracted from the relative fluorescence intensities of all the other solutions.

The concentration of BHA was determined as follows. The standard addition curve for PG was prepared by plotting the corrected relative fluorescence intensities obtained from the unknown solution and the three solutions spiked with PG against μg per ml of PG added to the lard solutions. The best straight line through these points provides a calibration curve with a slope representative of the fluorescence intensity of PG alone. An intensity value corresponding to the previously determined PG concentration is obtained from this curve and subtraction of the unknown intensity from this value gives the absolute intensity due to PG. The latter value is then subtracted from the intensities obtained from the unknown and the three BHA standard addition solutions and these values are plotted against μg per ml of BHA added to the unknown. Extrapolation of the best straight line through these points to zero relative intensity gives the concentration of BHA in the unknown solution.

DETERMINATION OF BHA IN CEREAL

Extraction of BHA. The method used was similar to that described by Schwecke and Nelson (1964). In this method both BHA and BHT are quantitatively extracted from cereal into ethyl ether. A 10.0-g sample of cereal was ground to a powder with a mortar and pestle and poured into a glass column 49.5 cm in length and 1.0 cm in diameter, which was fitted with a course fritted glass disc. (For spiked samples an aliquot of a 1000 μg per ml or 100 μg per ml solution of BHA in ethanol was added directly to the dry cereal in the column.) Ethyl ether was percolated through the column until 25 ml were collected in a 25-ml Erlenmeyer flask. The 25 ml of eluent were evaporated to dryness with a stream of nitrogen. Two milliliters of ethyl ether were added to the dried residue and added to a 5-ml volumetric flask. The Erlenmeyer flask was rinsed and the rinsings were added to the 5-ml volumetric flask and diluted to 5 ml with ethyl ether.

Thin-Layer Preparation and Sample Spotting. A thin layer of silica gel-G (20 cm × 10 cm × 0.20 mm) was applied to the thin-layer plate. The slurry of silica gel-G used for spreading, was prepared and applied as described by the manufacturer. After the plate was coated with silica gel-G, it was dried at room temperature and then activated at 110° C for 30 min. The plate was cooled to room temperature and four aliquots of the extract were applied to the plate. The aliquots were spaced on the plate using a template. A 50-μl aliquot of the extract was applied on the far right of the plate. A similar 50-μl aliquot was applied next to the first. On top of the second aliquot 5 μl of 1000 μg per ml of BHA in absolute ethanol was applied. Next to this spot a 5-μl aliquot of the same BHA solution was applied. Finally, another 50-μl aliquot of the extract was applied on the far left side of the plate.

Spot Development. The sample plate was then placed in a developing tank containing mobile solvent (200 ml of ligroine, 20 ml of ethanol, and 3 ml of acetic acid) and developed twice to a solvent front height of 10 cm.

Spot Detection. The first three spots applied were covered while the spot on the far left of the plate was sprayed with N,2,6-trichloro-*p*-benzoquinoneimine reagent. BHA showed up as a pink spot if a small amount was present, or as a blue spot for a larger amount. Three circular areas of 1.7 cm diameter corresponding to the location of BHA on the sprayed area were scraped from the unsprayed area into three 25-ml volumetric flasks fitted with small glass funnels. A blank was obtained by scraping off an area where no sample had migrated. A constant area for each spot was obtained by making an impression on the plate with a metal cylinder of 1.7 cm diameter. Next, 10.00 ml of absolute ethanol were added to the four 25-ml volumetric flasks. Each flask was vigorously hand-shaken for 3 min and decanted into test tubes. After centrifuging, relative fluorescence intensities were obtained from the four clear supernatants using a 1P28 phototube and slit arrangement four. The excitation monochromator was set at 290 nm and the emission monochromator was set at 327 nm. The relative fluorescence intensity obtained from the blank solution was subtracted from the other three.

Calculation of Parts Per Million BHA. The concentration of BHA is obtained using the following expression:

$$\text{BHA} = \text{R.I.U.}/\text{R.I.M.} - \text{R.I.U.} \times 0.500\ \mu\text{g/ml}$$

where R.I.U. = relative intensity of the unknown, R.I.M. = relative intensity of the mixture of standard and unknown, and 0.500 μg/ml = concentration of standard. The relative intensity of the standard by itself is used to monitor the chromatographic procedure and should have a value reasonably close to the difference between the mixture and the unknown.

If 10.00 g of cereal were used in the determination, the μg per ml of BHA was multiplied by 100 to obtain parts per million of BHA.

DETERMINATION OF BHA IN WAXED PACKAGING MATERIAL

Low Fluorescence Background. Some packaging materials are found to have a very low fluorescence background; there-

fore, the following procedure was used. A 0.5000 g sample of waxed paper obtained by punching holes with a paper punch was placed in a 100-ml beaker. Approximately 50 ml of ligroine were added to the beaker and the contents of the beaker were stirred for 30 min on a magnetic stirrer. (For spiked samples, aliquots of a 100 μg per ml BHA solution in ligroine were added directly to the beaker.) The solution was transferred to a 100-ml volumetric flask, and the paper in the beaker was rinsed four times with 10-ml aliquots of ligroine. The rinsings were added to the 100-ml volumetric flask, and the flask was diluted to volume. Ten milliliter aliquots of this solution were added to two 25-ml volumetric flasks. A 2.0-ml aliquot containing 10.0 μg per ml of BHA in 50% ethanol-ligroine was added to one flask and both were then diluted to volume with 50% ethanol-ligroine. Ten milliliters of ligroine were added to two other 25-ml volumetric flasks. One flask was diluted to 25 ml with 50% ethanol-ligroine, and to the other 2.0 ml of 10.0 μg per ml of BHA in 50% ethanol-ligroine were added and then diluted to 25 ml with 50% ethanol-ligroine. Relative fluorescence intensities were then obtained for all solutions with the excitation monochromator at 290 nm and the emission monochromator at 327 nm. The fluorescence intensity was detected with a 1P28 phototube using slit arrangement four. The relative fluorescence intensity from the blank solution was subtracted from the other three solutions.

Calculation of Percent BHA. The calculations were similar to those for cereal, except the ratios of relative intensities were multiplied by 0.800 μg per ml of BHA and the μg per ml of BHA was multiplied by 0.050 to obtain a percentage based on a 0.5000-g sample of packaging material.

High Fluorescence Background. For packaging material with high fluorescence background, the initial extract was evaporated to less than 2 ml with a stream of nitrogen. The extract was then added to a 10-ml volumetric flask. The beaker was rinsed with ligroine and the rinsings were added to the 10-ml volumetric flask.

The BHA was then separated by the thin-layer chromatographic procedure described above, except for the following modifications. Only 2 μl of 1000 μg per ml of BHA standard were spotted, the plate was developed only once, and 5 ml of ethanol were used to elute the spots.

Calculation of Percent BHA in Packaging Material. The calculations were similar to those for "low fluorescence background" except the ratios of relative intensities were multiplied by 0.400 μg per ml of BHA and the μg per ml of BHA was multiplied by 0.200.

RESULTS AND DISCUSSION

Determination of BHA. Lard. The advantages of using fluorescence for the determination of antioxidants in lard has been demonstrated by Latz and Hurtubise (1969). They used chloroform as a solvent to quench the fluorescence of BHA and BHT, which enabled them to determine PG in lard without prior separation. The determination of BHA in lard has also been accomplished without separation by using 50% ethanol-ligroine as a solvent, in which lard and all of the antioxidants are soluble. Also, the fluorescence intensity of BHA is increased by a factor of three in 50% ethanol-ligroine, as compared to BHA in ligroine. In 50% ethanol-ligroine, the fluorescence intensity of BHT is very low, and the maximum emission wavelengths of BHA and PG are separated by 30 nm. Because of these factors, BHA can be determined with BHT and PG present in solution. Problems with colorimetric methods such as differences in reaction rates of the isomers of BHA with a color reagent and differences in molar absorptivities of the final color complex do not occur with fluorimetric methods (Johnson, 1967; Mahon and Chapman, 1951, 1952). These problems are eliminated because a solution of the isomers gives only one maximum emission wavelength. The relative intensity at the maximum emission wavelength is used to obtain an accurate determination of BHA.

Table I. Determination of BHA in Lard Samples by Standard Addition

No. of Determinations	Added, ppm BHA	Added, ppm BHT	Added, ppm PG	BHA found, ppm	Error, %
4	120	...	40	128	+ 6.67
3	24.0	...	12.0	27	+12.5
2	10.0	...	30.0	14	+40.0
2	120	240	40.0	126	+ 5.00
2	40.0	40.0	...	40.0	0.00
2	80.0	240	...	84.5	+ 5.62
3	120	120	40.0	125	+ 4.17
3	80	...	...	81.7	+ 2.12

Table I shows the results for a number of determinations of BHA in spiked lard samples. The concentration of PG was assumed to be the same as added to the lard solutions. For all solutions measured, a blank relative fluorescence intensity value was subtracted. The blank value was obtained from an antioxidant-free lard solution. The background emission from four different antioxidant-free lard solutions was found to be similar. The average value of the relative fluorescence intensity of the lard solutions was 0.35. The relative fluorescence intensity of a typical unknown solution was 6.40; therefore, the background emission would contribute about 5.47% to the total fluorescence intensity. If a blank value is not subtracted, an error of about 10.0% or greater can be introduced in the determination of BHA. The results in Table I indicate that with other antioxidants present and when a blank value is subtracted, the percentage error is not very large.

Standard addition has several advantages. These advantages have been discussed by Latz and Hurtubise (1969). One disadvantage of the standard addition method in this work was that the maximum excitation and emission wavelengths of PG are not used to obtain the standard addition plot for PG. Because of this any small error in relative fluorescence intensity will be magnified, since the slope of the standard addition curve does not have a maximum value.

Cereal. Two separation steps were necessary for the determination of BHA in cereal because of interfering fluorescent and phosphorescent components. The initial extraction of antioxidant-free cereals yielded solutions that contained components whose fluorescence and phosphorescence emission wavelengths were similar to BHA and thus would interfere with the determination of BHA. Therefore, luminescence analysis of the eluent from the extraction column could not be used to determine BHA. Fluorescence was used to determine BHA after scraping from a thin-layer plate rather than phosphorescence because the fluorescence intensity of BHA is much greater than its phosphorescence intensity (Latz and Hurtubise, 1969).

If BHT is present in a cereal sample, it will not interfere in the determination of BHA because BHT moves with the solvent front on the thin-layer plate. The isomers of BHA which migrated at the same rate appeared as one pink or blue spot and had an average R_f value of 0.42. Thin-layer plates were

Sample	BHA added, ppm	BHA found, ppm	Recovery, %
Wheat	50.0[a]	47.7	95.6
	50.0[a]	55.0	110
	10.0	7.00	70.0
	10.0	9.56	95.6
Corn	50.0	51.8	103
	50.0	50.0	100
	10.0	11.6	116
	10.0	8.66	86.6
Rice	50.0	47.8	95.8
	50.0	48.8	96.4
	10.0	8.85	88.5
	10.0	8.59	85.9

[a] 20 μl of extract spotted.

Table III. Determination of BHA in Spiked Extracts from Waxed Paper with Low Fluorescence Background

BHA, added, %	BHA found, % blank not subtracted[a]	BHA found, % blank subtracted
0.100	0.110	0.105
0.100	0.114	0.111
0.050	0.051	0.048
0.050	0.056	0.051

[a] Blank solution, 10 ml of antioxidant free extract in ligroine diluted to 25 ml with 50% ethanol-ligroine.

developed twice with mobile solvent because with one development some cereal extracts showed a blue spot on the plate corresponding to almost the same position as BHA. However, two developments allowed BHA to be distinguished, and BHA always appeared above the unknown spot.

When the experimental procedure was carried out on BHA-free extracts of corn, rice, and wheat cereals, there was no background interference in almost all samples. With some samples a very small background fluorescence was observed, but it was not intense enough to cause error.

Table II shows that the method for determining BHA is sensitive and reasonably accurate in the part per million range. Also the method is generally applicable to corn, rice, and wheat cereals. The lower percent recovery at the 10 ppm level most likely results from the use of two separation steps for such a small amount of material. Recovery studies indicate that the initial cereal extraction is efficient using the procedure described, so it is suspected that the second extraction from silica gel is the primary source of error at the lower concentration levels. Repetitive determinations of BHA in three commercial samples of cereals containing this antioxidant produced results of 3.8 ± 0.4, 0.98 ± 0.6, and 2.0 ± 0.2 ppm. It was necessary to use 100 μl of concentrated extract for thin-layer separation to obtain these results and recovery experience suggests that the low values are most

(Stuckey and Osborne, 1965).

The method of spotting the different aliquots on thin-layer plates was chosen because this allowed the detection of errors such as uneven migration on the thin-layer plate or inefficient scraping of BHA from the plate. For example, if there was a large difference between the relative fluorescence intensity of the standard sample and the difference between the relative intensity of the unknown and standard addition sample, then it was assumed that some error, such as uneven migration on the thin-layer plate, had occurred. The method used to calculate the amount of BHA was employed because it corrected for any increase or decrease in the fluorescence emission of BHA that might result from some component present in the cereal extract or a component eluted from the silica gel.

Packaging Material. Table III gives the results for the determination of BHA from extracts of waxed paper that had a low fluorescence background. The results show that BHA can be accurately determined by a simple extraction procedure. However, with other antioxidant-free packaging materials, high fluorescence background was obtained and an accurate determination of BHA by simple extraction was impossible. Therefore, it is necessary to know beforehand the magnitude of the background fluorescence. Also, the glue used to seal the packaging material may be highly fluorescent; therefore, the sample of waxed paper used to determine BHA should not contain any traces of glue. In some cases the extracts of waxed paper were slightly cloudy, but this did not interfere with the determination.

Because some of the extracts from waxed paper had a high fluorescence background, it was again necessary to use thin-layer chromatography to separate BHA following extraction. Duplicate determinations of BHA in commercial waxed paper were performed using extraction alone and extraction combined with a thin-layer separation. The two approaches produced results of 230 and 60 ppm, respectively, which provides an indication of the extent of interference. The latter result is lower than might be expected, and although poor recovery might be partially responsible, it is felt that volatilization during storage may have occurred. Samples of freshly prepared wax paper were not available for verification.

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wash is colorless. Discard the isopropyl ether washes.

Acidify the NaOH solution with HCl. Add 25 ml iso-octane and shake 15–20 sec. Transfer the aqueous phase to a clean funnel and extract with 10 ml iso-octane. Repeat these extractions until an iso-octane wash is colorless. (If the isopropyl ether is practical grade, the aqueous phase may remain yellow.)

Wash the combined iso-octane extracts twice by shaking with 20–25 ml water. Drain as much water as possible from the funnel after the final wash. Pass the iso-octane solution thru a plug of absorbent cotton (previously washed with iso-octane). Wash the separatory funnel with about 20 ml iso-octane and pass this wash thru the cotton. If any color remains in the cotton, wash it with small portions of iso-octane to remove the color. Transfer the iso-octane solution to a 100 ml volumetric flask and make to volume with iso-octane. Determine the absorbance at 249 mμ in a 1 cm cell against a blank containing iso-octane. Check the reagents by running a reagent blank.

Results and Discussion

Table 1 gives recoveries of quinizarin when added to three samples of D&C Violet No. 2 at the 0.3 and 0.5% levels and recoveries of 1-hydroxyanthraquinone when added to three samples of D&C Violet No. 2 at the 0.2, 0.3, and 0.5% levels.

Commercial samples of D&C Violet No. 2 contained as much as 0.35% quinizarin or 1.0% 1-hydroxyanthraquinone. However, no sample contained both intermediates.

Table 1. Recoveries of 1-hydroxyanthraquinone and quinizarin added to 100 mg D&C Violet No. 2

Sample	Added, mg	Recovered mg	Recovered %
1-Hydroxyanthraquinone			
A	0.2	0.2	100
A	0.5	0.49	98
B	0.2	0.19	95
C	0.3	0.285	95
Quinizarin			
A	0.5	0.5	100
B	0.5	0.475	95
C	0.3	0.31	103

The procedure given here is applicable to either intermediate. The intermediate is readily identified by the color formed in aqueous alkaline solution. The absorptivities of both intermediates at 249 mμ are reasonably similar and, if both should be present, calculations based on either intermediate will give satisfactory determination of combined quinizarin and 1-hydroxyanthraquinone. However, since the manufacturing process determines which intermediate will be present in the color, both intermediates are not expected to be found in significant quantities in the same sample. Therefore, separation of these compounds was not attempted.

OILS, FATS, AND WAXES

Spectrophotometric Determination of BHA and BHT in Vegetable Oils

By D. P. JOHNSON (Research Department, R. J. Reynolds Tobacco Co., Winston-Salem, N.C. 27101)

Improved spectrophotometric methods have been developed for separate determinations of BHA and BHT in vegetable oils. The methods are applicable in the presence of other synthetic antioxidants and are adaptable to BHA and BHT concentrations ranging from 5 to 200 ppm. BHA is nitrosated directly in the oil and the nitroso product is isolated by a combination of solvent extraction and liquid chromatographic techniques. An alkaline solution of the product is measured spectrophotometrically at 480 mμ, and the absorbance is referred to a calibration curve to determine the BHA concentration. Both the 2- and 3-isomers yield approximately the same

absorptivity, and Beer's Law is obeyed from 0 to 50 μg BHA/ml final solution. BHT is determined by its ultraviolet absorption at 283 mμ. The compound is separated from the oil by direct extraction into acetonitrile. BHT is isolated from BHA and other extraneous materials by chromatography on alumina.

Methods available for determining antioxidants in food products generally are based on the phenolic and reducing properties of the compounds and often lack the sensitivity and specificity needed for measuring ppm concentrations. The development of methods meeting these requirements is complicated both by the number and chemical similarity of the synthetic antioxidants currently being used and by the complex nature of the biological substrates to which they are added. The problem is further intensified by the practice of using the antioxidants in combination as permitted by governmental control agencies.

The most commonly used food grade antioxidants include butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG), and nordihydroguaiaretic acid (NDGA). All of these compounds react with ferric chloride and 2,2-bipyridine to form a red color which may be used for their combined determination (1). Since NDGA reacts at a different rate from the other antioxidants, it can be estimated independently by close control of the reaction time and by the use of simultaneous equations for the calculation (2). BHA may be estimated by the intensity of the blue color produced when it is reacted with 2,6-dichloroquinonechlorimide (2). Thus, by using a combination of the ferric chloride-bipyridine and the 2,6-dichloroquinonechlorimide methods, individual estimates can be made for BHA, BHT, and NDGA in mixtures.

Propyl gallate, like other trihydroxyphenols, forms a complex with ferrous tartrate which yields a colored solution. The intensity of the color is proportional to the PG concentration and has been adapted to a quantitative measurement of that compound (2). NDGA also reacts with ferrous tartrate, but the product is not soluble in the solvent system and therefore does not interfere with the PG analysis.

Szalkowski and Garber (3) described the only known specific colorimetric method for BHT. The method is based on reaction of BHT with dianisidine and nitrous acid and reportedly is satisfactory for concentrations ranging from 10 to 200 ppm. Other methods for BHT are based on its absorption in the ultraviolet (UV) range (4, 5) and on gas-liquid chromatography (6).

Many of the methods available for antioxidants have limitations that restrict their utility. For example, the reaction of these substances with ferric chloride and bipyridine is subject to interference from almost any other reducing material. The color formed in the Szalkowski and Garber method for BHT is highly sensitive to light, showing rapid deterioration upon exposure. Consequently, the analysis must be performed without delay and in subdued light. Even though the 2,6-dichloroquinonechlorimide method is selective for BHA, the two isomers of that compound respond differently, with the 3-isomer yielding about four times as much color as the 2-isomer (1). Although the 3-isomer constitutes 85–90% of technical BHA, the isomers respond to oxidation at different rates and the ratio may be changed upon prolonged exposure to oxidative conditions. Sahasrabudhe (7) avoided many of these problems by using thin layer chromatography to isolate the individual antioxidants prior to colorimetric analysis. However, the latter procedure was designed for concentrations approximating the maximum allowed by the FDA and in some cases, it may not possess the sensitivity needed for studying the rate of degradation of the antioxidants in used oil.

Because of the limitations of existing methods, an investigation was undertaken to provide procedures for BHA and BHT that would be both reliable and adaptable to a wide range of concentrations. Cottonseed oil was chosen as the experimental substrate because it is a complex biological medium and because it is representative of vegetable oils that frequently are stabilized with these antioxidants. Emphasis was

placed both on rapid and efficient means of separating the antioxidants from the oil and on sensitive end-determinations for BHA and BHT in the presence of other antioxidants.

BHA METHOD

Apparatus and Reagents

(a) *Florisil.*—60-100 mesh, with ca 3% "apparent" water content as determined by titration with Karl Fischer reagent.

(b) *Petroleum ether:acetone mixture.*—Mix 100 ml reagent grade acetone with 900 ml petroleum ether.

(c) *Sodium nitrite.*—2% aqueous solution; prepare fresh weekly.

(d) *Hydrochloric acid.*—About 3*N*.

(e) *Sodium hydroxide.*—Dissolve 40 g NaOH in 500 ml water and add 500 ml methanol. Mix and adjust volume to 1 L with water.

(f) *Cottenseed oil.*—Free of antioxidants.

(g) *Spectrophotometer.*—Beckman Model B, or equivalent.

(h) *Chromatographic column.*—20 x 400 mm chromatographic tube; add 1½" Florisil and top with ½" anhydrous sodium sulfate.

Procedure

Transfer 10 g oil sample to 125 ml separatory funnel and add 50 ml petroleum ether: acetone solution. Add 5 ml sodium nitrite solution and 5 ml 3*N* HCl. Stopper immediately and shake vigorously 30 sec. Let phases separate completely and discard lower layer. Extract organic layer with 20 and 5 ml portions of NaOH solution, respectively, shaking well for 30 sec during each extraction. Combine extracts in a 125 ml separatory funnel containing 15 ml 3*N* HCl. Extract aqueous solution twice with 25 ml portions of petroleum ether:acetone mixture, pass each extract individually through Florisil column, and collect filtrate in 250 ml Erlenmeyer flask. When last of second extract has entered surface of column, add 25 ml more of petroleum ether:acetone solution. Transfer eluate to 125 ml separatory funnel and add, with pipet, 20 ml NaOH solution. Shake 30 sec and let phases separate. Transfer portion of lower layer to 1 cm cell and, using NaOH solution to zero spectrophotometer, measure absorbance at 480 mμ. Refer absorbance to calibration curve to determine μg BHA present. Calculate ppm BHA as follows:

$$\text{ppm BHA} = \mu\text{g BHA/g sample}$$

Calibration Curve

Prepare standard solution containing 100 μg inhibitor grade BHA/ml petroleum ether: acetone mixture. To four separate 125 ml separatory funnels, add 10 g uninhibited cottonseed oil. Add, respectively, 1, 2, 5, and 10 ml standard BHA solution and 49, 48, 45, and 40 ml petroleum ether:acetone mixture. Process these solutions as described in procedure, beginning with the addition of sodium nitrite and HCl. Using NaOH solution to zero spectrophotometer, determine absorbance of standards at 480 mμ. Prepare calibration curve by plotting absorbance values vs. μg BHA in samples.

BHT METHOD

Apparatus and Reagents

(a) *Pentane.*—Colorless, analytical grade

(b) *2,2,4-Trimethylpentane (iso-octane)*

(c) *Acetonitrile.*—Commercial grade; distill through unpacked column, discarding small heads and tails fractions.

(d) *Sodium chloride.*—2% aqueous solution.

(e) *Alumina, acid.*—Woelm (Alupharm Chemicals, 610 Commercial Place, New Orleans, La. 70130); adjust "apparent" water content to 3.00% as follows: Determine initial water content by titration with Fischer reagent and add enough water (usually ca 2.5%) to yield a concentration of 3%. Mix alumina by rolling or other means for 2 hr before use.

(f) *Chromatographic column.*—Add 1½' alumina to 20 × 400 mm chromatographic tube and then add ¼" anhydrous sodium sulfate.

(g) *Spectrophotometer, recording.*—Beckman Model DB, or equivalent.

Procedure

Transfer 25 g oil to 125 ml separatory funnel designed for centrifugation. (*Note:* If sample is solid, heat it in hot water bath or oven at 100°C until it melts.) Add 50 ml acetonitrile and shake vigorously 1 min. Centrifuge mixture 5 min at 1200 rpm. (If a centrifuge is not readily available, the layers may be allowed to separate by gravity.) Transfer oil layer to another centrifuge funnel and decant acetonitrile phase into 500 ml separatory funnel; do not let any of residual oil flow into funnel. Repeat extraction with two additional 50 ml portions of acetonitrile and add 200 ml NaCl solution to funnel containing the combined extracts. Extract mixture with two 50 ml portions of pentane, shaking well for 30 sec each time. Filter pentane ex-

tracts into 250 ml Erlen
small bed of sodium sulf
funnel fitted with glass wool
pentane with vacuum to ca
ml iso-octane. Transfer solu
tographic column, using 5
rinse flask. Collect eluate in
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enter surface of column and
another 5 ml portion of is
rinse solution to column.
octane to column to perm
exactly 50 ml eluate. Mix
transfer portion to 1 cm q
iso-octane previously pass
alumina column to zero s
measure absorbance at 283
ance to calibration curve to d
in sample. (*Note:* If possible
from 320 to 210 mμ. The cha
at 283 and 276 mμ is posi
BHT.) Calculate ppm BHT

ppm BHT = μg BHT

Calibration Curve

Prepare standard solutio
μg BHT/ml acetonitrile. To
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standard solution, respectiv
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then add ca 25 g unin
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Results and Dis

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separate by gravity.)
another centrifuge fun-
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Repeat extraction with
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tracts into 250 ml Erlenmeyer flask through small bed of sodium sulfate contained in 60° funnel fitted with glass wool plug. Evaporate pentane with vacuum to ca 2 ml and add 10 ml iso-octane. Transfer solution to chromatographic column, using 5 ml iso-octane to rinse flask. Collect eluate in 50 ml volumetric flask fitted with small funnel. Let sample enter surface of column and rinse flask with another 5 ml portion of iso-octane, adding rinse solution to column. Add enough iso-octane to column to permit collection of exactly 50 ml eluate. Mix eluate well and transfer portion to 1 cm quartz cell. Using iso-octane previously passed through an alumina column to zero spectrophotometer, measure absorbance at 283 mμ. Refer absorbance to calibration curve to determine μg BHT in sample. (*Note:* If possible, record spectrum from 320 to 240 mμ. The characteristic doublet at 283 and 276 mμ is positive evidence for BHT.) Calculate ppm BHT as follows:

$$\text{ppm BHT} = \mu\text{g BHT/g sample}$$

Calibration Curve

Prepare standard solution containing 100 μg BHT/ml acetonitrile. To four separate 125 ml separatory funnels designed for centrifugation, pipet 2, 5, 10, and 20 ml aliquots of standard solution, respectively. Add 48, 45, 40, and 30 ml acetonitrile, respectively, and then add ca 25 g uninhibited cottonseed oil to each. Process standards as described in *Procedure*, beginning with ". . . shake vigorously 1 min." Prepare calibration curve by plotting absorbance values vs. μg BHT in samples.

Results and Discussion

The open *ortho* position in the BHA molecule suggested that this compound would react with nitrous acid to form a colored nitroso derivative. Although the 3-isomer has both the 2 and 5 positions open, it was suspected that the tertiary butyl group on the number 3 carbon would hinder or prevent nitrosation at the number 2 position, thereby resulting in approximately the same colorimetric response for both isomers. BHT, with both the *ortho* and *para* positions occupied, was not expected to form a colored nitroso product. Although PG would be susceptible to nitrosation, its water solubility offered a means of separating that compound from BHA prior to the reaction.

To test the response of the antioxidants to nitrosation, solutions of inhibitor grade BHA, BHT, and PG in chloroform were shaken with sodium nitrite and hydrochloric acid. As expected, BHA produced a yellow color which exhibited maximum absorption at 420 mμ. PG produced a red color that faded rapidly, while the BHT solution remained colorless. By substituting petroleum ether for chloroform and extracting the solutions with water prior to nitrosation, only BHA produced color.

Nitrosation of BHA for analytical purposes is conducted directly in the cottonseed oil. However, the natural color of the oil interferes with a direct colorimetric measurement of the product. Also, certain oil constituents, possibly sterols, form nitroso products which absorb in the same general region as BHA. To eliminate these interferences, the sample is extracted with sodium hydroxide to eliminate the oil, and after acidification, it is re-extracted into petroleum ether:acetone for the chromatographic cleanup with Florisil. The BHA product is recovered in the eluate while the nitrosated oil products are retained by the adsorbent. For further insurance against interferences, and to increase the sensitivity of the method, the BHA product is transferred to an alkaline solution for colorimetric measurement. Under these conditions, maximum absorption occurs at 480 mμ, and Beer's Law is followed over the range of 0 to 50 μg BHA/ml solution.

In addition to removing natural interferences, the Florisil removes PG from the sample solution, making a separate water extraction unnecessary.

Tests showed that maximum nitrosation of BHA could not be achieved in petroleum ether solution alone. The presence of cottonseed oil in the solution significantly enhanced the reaction but it still did not yield maximum color. It was suspected that the reaction was hindered by the lack of solubility of the aqueous reactants in the organic solution, and to test this hypothesis, samples of BHA in petroleum ether solution were analyzed with increasing amounts of acetone added to serve as co-solvent. The effects are illustrated in Fig. 1 which shows that maxi-

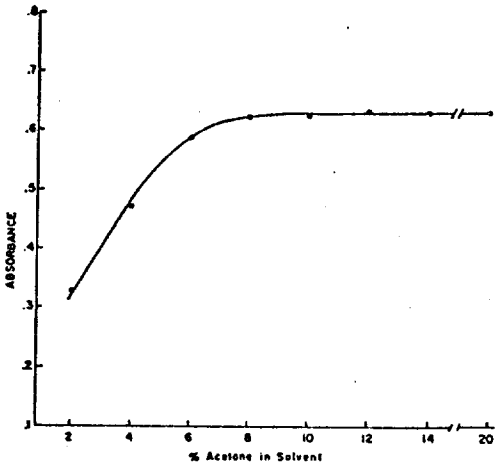

Fig. 1—Effect of acetone concentration on nitrosation of BHA.

mum reaction is obtained when 10% or more of acetone is present. Even in the presence of acetone, however, cottonseed oil enhanced the color to a slight degree, and for that reason, it is recommended that uninhibited oil be included in the standards when preparing the calibration curve.

The 2- and 3-isomers of BHA were checked separately for response to the nitrosation reaction. The absorbance-wavelength curves, which are illustrated in Fig. 2, show approximately the same absorptivity for the two isomers, although the absorbance maximum for the 3-isomer appears 15 mμ higher than that for the 2-isomer. Because of the predominance of the 3-isomer in commercial BHA, the maximum for that isomer was selected for calibrating the present method. Under these conditions, the error for the 2-isomer is less than 5%.

Data showing the recovery of various concentrations of BHA from different oils are given in Table 1. Recoveries averaged 99.4 ± 0.9% over a range of 5 to 70 ppm. The data show that neither BHT nor PG interfere in the analysis. Based on the average absorbance value for the control samples, the method is sensitive to at least 2 ppm.

With both the *ortho* and *para* positions blocked, BHT is not subject to the usual nitrosation and diazotization reactions that impart color to phenolic compounds. However, BHT absorbs strongly in the UV, as

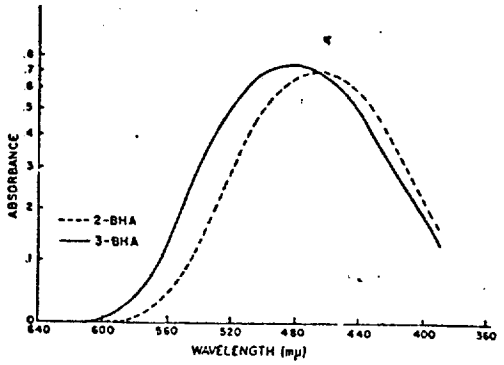

Fig. 2—Absorption spectra of the isomers of BHA.

Table 1. Recovery of BHA from vegetable oils

Oil	BHA Added, ppm	Absorbance	Recovery, %	Dev. from Mean, %
Cottonseed	0	0.004	—	—
	0	0.001	—	—
	20	0.320[a]	100.00	+0.54
	20	0.320	100.00	+0.54
	50	0.779[a]	98.40	−1.06
	50	0.780[b]	99.00	−0.46
Corn	0	0.005	—	—
	0	0.008	—	—
	50	0.770	97.40	−2.06
	50	0.785	100.00	+0.54
Soybean	0	0.003	—	—
	5	0.081	101.40	+1.94
	30	0.472[b]	100.00	+0.54
	70	1.070	97.14	−2.32
Hydrogenated vegetable	0	0.000	—	—
	10	0.159	100.00	+0.54
	30	0.473	100.21	+0.75
	50	0.785	100.00	+0.54
		Av.	99.46	±0.99

[a] BHT present at same concentration as BHA.
[b] PG present at same concentration as BHA.

shown in Fig. 3, forming a doublet with maxima at 283 and 276 mμ. The absorptivity at both wavelengths is essentially the same, and Beer's Law is obeyed from 0 to 40 μg BHT/ml solution.

Separation of BHT from the oil for UV measurement is accomplished by direct extraction into acetonitrile. The antioxidant is then extracted into pentane after sodium chloride solution is added to the acetonitrile to reduce the solubility of the antioxidant. Final isolation of BHT from extraneous oil extractives, as well as from BHA and PG if present, is achieved by chromatography on alumina.

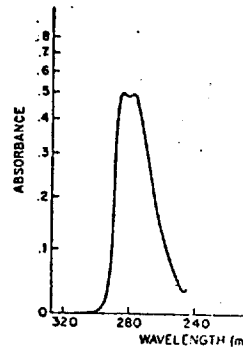

Fig. 3—Ultraviolet spectr

Efforts to use various co
of alumina resulted in
Different lots from the sa
different recoveries and att
the activity were only par
However, Woelm acid-type
be standardized satisfactoril
was recommended.

Data showing recoveries
various oils at concentratio
8 to 80 ppm are listed in
BHA nor PG, when pr
tions as high as 100 ppm.,
analysis. Based on the ave
value of the uninhibited
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Somewhat greater sensitivi
tained either by increasing
or by concentrating the
chromatographic column.

The characteristic shape o
trum provides positive ident
compound, even at low
Therefore, it is recomme
spectrum be recorded when
tions of BHT are being d
allows more positive distinct
antioxidant and possible bac
tion. If any absorption ap
lengths above 300 mμ, int
be suspected. In this case, a
be drawn between 300 and
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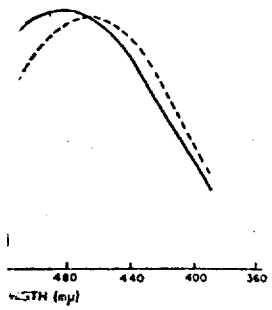
technique must also be used
tion of the calibration curv

The BHT method was suc
to oils from several sources



a of the isomers of BHA.

BHA from vegetable oils

bsorb-ance	Recov-ery, %	Dev. from Mean, %
0.004	—	—
0.001	—	—
0.320[a]	100.00	+0.54
0.320	100.00	+0.54
0.779[a]	98.40	−1.06
0.780[b]	99.00	−0.46
0.0	—	—
0.(	—	—
0.7	97.40	−2.05
0.785	100.00	+0.54
0.003	—	—
0.081	101.40	+1.94
0.472[b]	100.00	+0.54
1.070	97.14	−2.32
0.000	—	—
0.159	100.00	+0.54
0.473	100.21	+0.75
0.785	100.00	+0.54
Av.	99.46	±0.99

concentration as BHA.
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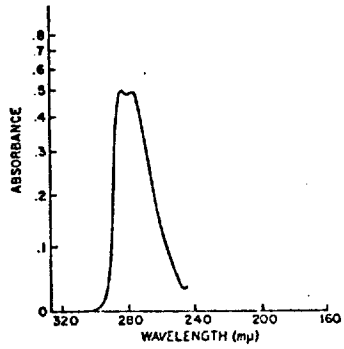

Fig. 3—Ultraviolet spectrum of BHT.

Efforts to use various commercial grades of alumina resulted in limited success. Different lots from the same source gave different recoveries and attempts to adjust the activity were only partially successful. However, Woelm acid-type alumina could be standardized satisfactorily and therefore was recommended.

Data showing recoveries of BHT from various oils at concentrations ranging from 8 to 80 ppm are listed in Table 2. Neither BHA nor PG, when present in concentrations as high as 100 ppm, interfered in the analysis. Based on the average absorbance value of the uninhibited oil samples, the sensitivity of the method is at least 4 ppm. Somewhat greater sensitivity can be obtained either by increasing the sample size or by concentrating the eluate from the chromatographic column.

The characteristic shape of the BHT spectrum provides positive identification of that compound, even at low concentrations. Therefore, it is recommended that the spectrum be recorded when low concentrations of BHT are being determined. This allows more positive distinction between the antioxidant and possible background absorption. If any absorption appears at wavelengths above 300 mμ, interference should be suspected. In this case, a baseline should be drawn between 300 and 245 mμ and the net absorbance measured at 283 mμ. Obviously, for these samples, the baseline technique must also be used in the preparation of the calibration curve.

The BHT method was successfully applied to oils from several sources and presumably

Table 2. Recovery of BHT from vegetable oils

Oil	BHT Added, ppm	Absorb-ance	Recov-ery, %	Dev. from Mean, %
Cottonseed	0	0.008	—	—
	0	0.006	—	—
	8	0.035	98.50	+0.52
	28	0.122	97.14	−0.84
	40	0.190	106.00	+8.02
	40	0.181	100.50	+2.52
	60	0.268	101.00	+3.02
	80	0.336	95.00	−2.98
Corn	20	0.095	106.00	+8.02
	40	0.180	100.00	+2.02
	80	0.350	98.50	+0.52
Soybean	0	0.002	—	—
	8	0.031	85.00	−12.98
	28	0.115	91.43	−6.55
	60	0.247	92.33	−5.65
Hydrogenated vegetable	0	0.009	—	—
	20	0.086	97.00	−0.98
	40	0.186	104.50	+6.52
	80	0.345	96.75	−1.23
		Av.	97.98	±4.16

can be adapted to others. Products that are solid at room temperature must be melted prior to analysis and may also require periodic heating during the extraction process to maintain the liquid state.

The BHA and BHT methods were used to study the rate of degradation of these antioxidants in heated cottonseed oil. In this manner, the capability of the methods to distinguish between the parent antioxidants and their oxidation products was demonstrated. A sample of oil containing 0.1% each of BHA and BHT was heated for 5 hours at 175°C with continuous agitation. Fractions were removed at 1-hour intervals and analyzed for the antioxidant content. The results are shown in Table 3 and indicate that only the parent compounds responded to the analyses, with no apparent interference from the degradation products.

Table 3. Rate of degradation of BHA and BHT in cottonseed oil at 175°C

Heating Time, hr	Recovery, %	
	BHA	BHT
0	102.10	96.12
1	86.78	71.31
2	70.96	60.05
3	53.41	36.70
4	37.80	21.82
5	17.55	12.09

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Sesquiterpene Hydrocarbons of Commercial Copaiba Balsam and American Cedarwood Oils

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The sesquiterpene hydrocarbons of commercial samples of copaiba balsam and American cedarwood oils were separated by a combination of techniques including silver nitrate adduction, column chromatography on alumina, distillation, and gas chromatography. Components were identified by their infrared spectra. Of the 24 sesquiterpene hydrocarbons detected in copaiba balsam oil, 18 were identified as follows: δ-elemene, copaene, β-cubebene, cyperene, α-bergamotene, β-elemene, caryophyllene, γ-elemene, β-farnesene, allo-aromadendrene, α-, β-humulene, β-bisabolene, α-selinene, β-selinene, δ-cadinene, ar-curcumene, γ-cadinene, calamenene, and α-cubebene (tentatively identified). The following sesquiterpene hydrocarbons were identified in American cedarwood oil: β-elemene, α-cedrene, caryophyllene, thujopsene, α-, β-humulene, "acorene," valencene, cuparene, a cuprenene, and two additional hydrocarbons (tentatively identified). Gas chromatographic retention data are reported for 27 of the sesquiterpene hydrocarbons isolated.

In a previous paper (1), we reported on the usefulness of sesquiterpene hydrocarbon analysis as a means of characterizing essential oils and detecting the presence of certain diluents. The present work attempts to more fully characterize the sesquiterpene hydrocarbons of commercial copaiba balsam and cedarwood oils, both of which are used as diluents for other perfume oils (2). Some of the isolated sesquiterpene hydrocarbons have also provided us with authentic reference standards for infrared (IR), nuclear magnetic resonance (NMR), and gas chromatographic (GLC) studies.

Sesquiterpene hydrocarbons from natural sources generally occur as complex mixtures difficult to separate even with the most advanced GLC techniques (3, 4). In our studies of the sesquiterpene hydrocarbon content of numerous essential oils, we frequently encountered mixtures of sesquiterpene hydrocarbons which could not be satisfactorily resolved by GLC, using packed columns and a variety of liquid phases.

In this report we outline a scheme for preliminary separation of the sesquiterpene hydrocarbons by distillation, silver nitrate adduction, and column chromatography. Various combinations of these techniques have been tried, but those reported here were found to be most suitable for the two oils studied. With these procedures, we were able to isolate and identify several individual components difficult or impossible to separate by packed-column GLC of the original hydrocarbon fractions; no decomposition or isomerization of the sesquiterpene hydrocarbons was noted.

The nomenclature of sesquiterpene hydrocarbons is not yet fully standardized. In order to avoid some of the difficulties we have encountered in the past, we used those

are the same, the solution given earlier[1] can be used in this case also.

T_2, the bulk temperature of the fluid flowing through a duct of length Z when heat transfer occurs from all three sides of the duct maintained at T_w may be obtained from the heat balance

$$\int_0^Z \int_0^{a/\sqrt{3}} -K\left(\frac{\partial T}{\partial \eta}\right)_{\eta=0} dy dz = \frac{a^2}{6\sqrt{3}} V\rho C(T_2-T_1) \quad ...(10)$$

In terms of the Laplace transform, Eq. (10) becomes

$$\frac{\alpha}{p}\int_0^{a/\sqrt{3}} \left(\frac{dg}{d\eta}\right)_{\eta=0} dy = -\frac{a^2}{6\sqrt{3}} V(g_2-T_1) \quad ...(11)$$

Substituting the values of f and b from Eqs. (5) and (7), and using the value of $(dg/d\eta)_{\eta=0}$ given earlier[2] in Eq. (11), we obtain

$$\frac{g_2-T_1}{T_w-T_1} = \frac{(45)^{1/3}\Gamma(2/3)[\beta(1/2, 4/3)]\alpha}{\Gamma(4/3)a^{4/3}V}\left(\frac{\beta V}{\alpha}\right)^{1/3} p^{-2/3} \quad ...(12)$$

where β (m, n) is the beta function.

On inversion of the transform, Eq. (12) becomes

$$\frac{T_2-T_1}{T_w-T_1} = \theta = \frac{3(45)^{1/3}[\beta(1/2, 4/3)]}{2\Gamma(4/3)}\left(\frac{\alpha}{aV}\right)^{2/3}\left(\frac{Z}{a}\right)^{2/3}\rho^{1/3} \quad ...(13)$$

The average film coefficient of heat transfer, h_i, based on the inlet temperature difference (T_w-T_1), may be obtained from the heat balance

$$h_i(T_w-T_1)6\frac{aZ}{\sqrt{3}} = \frac{a^2}{\sqrt{3}} V\rho C(T_2-T_1) \quad ...(14)$$

The velocity distortion factor, ρ, can be taken as

$$\rho^{1/3} = B\left(\frac{\mu_a}{\mu_w}\right)^{0.14} \quad ...(15)$$

Using a value of 1·15 for the constant B in the absence of precise experimental data[5], combining Eqs. (13)-(15) and rearranging the terms it can be shown that

$$j_h = \left(\frac{h_i}{CV\rho}\right)\left(\frac{C\mu_a}{K}\right)^{2/3}\left(\frac{\mu_w}{\mu_a}\right)^{0.14} = 1.68\left(\frac{D_e V\rho}{\mu_a}\right)^{2/3}\left(\frac{Z}{D_e}\right)^{-1/3} \quad ...(16)$$

where $D_e = [(2/3)a]$, the equivalent diameter.

Eq. (16) holds good even if only one side or one half of a side from a corner is heated. The Colburn correction[6] for natural convection effects may be applied to Eq. (16) when it is necessary.

The local heat transfer coefficient, h_i°, based on the inlet temperature difference, T_w-T_1, may be shown to be

$$\left(\frac{h_i^\circ D_e}{K}\right) = 1.33\left(\frac{D_e V\rho}{\mu_a}\right)^{1/3}\left(\frac{C\mu_a}{K}\right)^{1/3}\left(\frac{z}{D_e}\right)^{-1/3} \times \left(\frac{\mu_a}{\mu_w}\right)^{0.14}\left[1-\left(\frac{y}{a/\sqrt{3}}\right)^2\right]^{1/3} \quad ...(17)$$

The more commonly used local film coefficient, h°, based on the wall and bulk temperature difference can be obtained as

$$h^\circ = h_i^\circ\left(\frac{1}{1-\theta}\right) \quad ...(18)$$

The value of θ, when all the three sides are heated, can be obtained from Eq. (13). When only half of a side from a corner is heated, one sixth of the value of θ computed from Eq. (13) is to be used in Eq. (18).

Nomenclature

a	= altitude of the triangle
C	= heat capacity of the fluid
j_h	= Colburn heat transfer factor
K	= thermal conductivity
p	= Laplace transform parameter
T	= temp.
u_z	= local velocity
V	= av. velocity
Z	= length of the duct
$\Gamma(x)$	= gamma function
μ_a, μ_w	= viscosities at av. and wall temp.
ρ	= density of the fluid

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Determination of Butylated Hydroxyanisole (BHA) & Butylated Hydroxytoluene (BHT) in Vitamin A Concentrates by Gas Liquid Chromatography

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A simple and rapid gas liquid chromatographic method is described for estimating butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) in vitamin A concentrates. Samples of vitamin A concentrates containing 0·1 per cent BHA and BHT can be analysed employing an Apiezon M (10 per cent) column with 100-120 mesh celite 545 as the inert support; a single analysis takes only 15 min. The method is free from interference from other compounds likely to be present. Recoveries of the order of 96-103 per cent of added BHA and BHT are obtained.

THE methods[1,2] currently used for estimating butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) in vitamin A concentrates involve the use of 72 per cent alcohol for the extraction of BHA and BHT and then reacting them with 2,6-dichloroquinone chloroimide for the determination of BHA and with ferric chloride and α,α′-dipyridyl for the determination of BHA and BHT. These methods are laborious and several

reducing substances give the same reaction with ferric chloride and α,α′-dipyridyl as BHT, and thus interfere with the estimation of BHT.

Some gas liquid chromatographic methods described in literature[3,6] for determining BHA and BHT in food products involve elaborate extractions prior to chromatography. The method described in this communication does not involve any preliminary extraction. It can be used for samples containing as little as 0·1 per cent of BHA and BHT and is not subject to interference from other compounds likely to be present. Once the apparatus is set up, the time taken for each determination is about 15 min. Determinations can be carried out in the range 0·10-3·0 per cent of the antioxidants, the range normally found in commercial vitamin A concentrates.

The apparatus used was a Pye argon gas chromatograph with a β-ray (strontium 90; 20 mC.) ionization detector. A glass column 4 ft in length and 4 mm. in diam. was used as the separating column. Of the various thermostable substances tried, Apiezon M (Metropolitan Vickers Electric Co. Ltd) and silicone elastomer (E301) (7·5 per cent) as the immobile phase gave satisfactory resolution.

Preparation of the column[7] — The inert support used for chromatography was 100-120 mesh celite 545. Enough hydrochloric acid (mixture of equal volumes of conc. HCl and water) was added to cover it in a beaker. The mixture was stirred slowly for 30 min. The supernatant acid was decanted and the celite washed 8-9 times with distilled water by stirring and decantation until the liquid was no longer acid to litmus. Then 25 ml. water were added and the *p*H of the slurry was adjusted to 7·0 (using a *p*H meter) by adding methanolic potassium hydroxide dropwise (0·5 g. in 100 ml. of 90 per cent methanol). It was then washed 4-5 times with water and dried at 110°C. in a vacuum oven (1 mm. Hg).

Apiezon M (1·0 g.) was dissolved in about 50 ml. of diethyl ether in a round-bottomed flask and 9·0 g. of dried celite added slowly while stirring. Ether was evaporated on a steam bath and the celite dried for 36 hr at 110°C. under reduced pressure as before. The celite prepared was packed into the column.

The operating conditions were: column, Apiezon M (10 per cent) on 100-120 mesh celite; column temp., 175°C.; flow rate, 70-80 ml./min.; sensitivity, X3; detector voltage, 1250 V.; chart speed, 12 in./hr; and load, 0·1 μl.

Calibration — Of the several internal standards tried, chlorocresol (BP) was found to be most suitable for calibrating the column. The calibration factor was determined by dissolving 40 mg. of chlorocresol, 30 mg. of BHA and 30 mg. of BHT in 1·0-1·5 ml. of light petroleum (100-120°C.) and injecting 0·1 μl. of the solution in the column. calibration factors (*Fc*) were determined before and after the experimental runs and were found to be reasonably constant.

$$Fc = \frac{\text{peak height of int. std}}{\text{wt of int. std}} \times \frac{\text{wt of pure sample (BHA and BHT)}}{\text{peak height of sample}}$$

Test sample run — In about 1 ml. of light petroleum, about 1·0 g. of vitamin A concentrate and about 40 mg. of chlorocresol, both accurately weighed, were dissolved and 0·1 μl. of the solution was injected into the column. Peak heights were noted and the percentage of BHA or BHT calculated as follows:

$$\text{BHA or BHT} = \frac{\text{peak height of BHA (or BHT)}}{\text{wt of concentrate}} \times \frac{\text{wt of internal std}}{\text{peak height of int. std}} \times 100 \times Fc$$

The column is not contaminated even after several injections, provided normal care of flushing the column at regular intervals is taken.

To judge the accuracy of the procedure, known amounts of pure BHA and BHT were added to samples of vitamin A palmitate and acetate of potencies 1·65 and 1·30 m.i.u./g. respectively and the mixtures analysed. It is seen from the results obtained (Table 1) that the recoveries are in the range 96·7-103·6 per cent.

TABLE 1 — RECOVERIES OF BHA AND BHT FROM VITAMIN A PALMITATE AND ACETATE

[The sample of vitamin A palmitate used contained originally 1·59 and 1·21 per cent (wt/wt) of BHA and BHT respectively. The corresponding figures for vitamin A acetate were 1·01 and 0·95 per cent]

BHA added % (wt/wt)	Total BHA found % (wt/wt)	Recovery %	BHT added % (wt/wt)	Total BHT found % (wt/wt)	Recovery %
VITAMIN A PALMITATE					
0·53	2·08	98·2	0·71	1·95	101·5
1·21	2·75	98·3	1·93	3·24	103·2
1·76	3·29	98·3	2·04	3·32	102·1
0·10	1·69	100·0	0·10	1·36	103·8
0·20	1·73	96·7	0·20	1·46	103·6
VITAMIN A ACETATE					
0·63	1·62	98·8	0·82	1·76	99·4
1·00	1·99	99·0	0·95	1·89	99·5
1·74	2·74	99·7	1·83	2·77	99·7

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Silica gel TLC does not throw any light on the homogeneity of individual *o*- or *p*-alkylphenols having different chain lengths. However, reversed-phase TLC, with the solvent system acetic acid–water (80:20), separates the *o*- or *p*-alkyl phenol homologues due to the difference in the chain length of the alkyl groups. Fig. 3 shows that this technique effectively separates dodecyl, tetradecyl, hexadecyl and octadecyl phenols among the *ortho* or *para* class of compounds (items A, B, C and D; G, H, I and J) but does not distinguish isomeric alkyl phenols (items A and J; B and I; C and H; D and G) which can be separated only by silica gel TLC (Fig. 2). These separations also indicate that resolution of some of the lower and higher homologues is possible. This method is useful in determining their purity. The results of all the separations are summarised in Table I.

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CHROM. 4220

Simple and rapid polyamide chromatography of antioxidants

The use of paper or thin-layer chromatography for the identification of antioxidants had been widely discussed[1-3]. A simple and rapid method for the analysis of antioxidants by polyamide layer chromatography is presented in this note. The results described are obtained with universally used spray reagents and two developing solvent systems.

Experimental

Chemicals. Eight kinds of antioxidant of F.D.A. standard were used. The solvents and chemicals are the first grade of Katayama Chemical Industries, Ltd., Osaka, Japan.

Thin-layer sheets. All the polyamide thin-layer sheets used were 15 × 15 cm and produced by Cheng Chin Trading Co. Ltd., Taipei, Taiwan.

Chromatography. The standard techniques of ascending thin-layer chromatography[4] was employed. The solvent systems were: petroleum ether (30–70°)–benzene–acetone (8:2:5) and acetone–ethyl alcohol–water (4:1:2).

The two spray reagent
spraying the first reagent th
with the second reagent.

(1) 5 % (v/v) bromide
tetrachloride.

(2) 0.25 % (w/v) fluore
in 5 ml of N,N-dimethylform

TABLE I

CHROMATOGRAPHIC DATA OF PURI
System (I) petroleum ether (30–
(4:1:2).

No.	Antioxidant
1	Butyl hydroxytoluene
2	Butyl hydroxyanisole
3	Stearyl gallate
4	Cetyl gallate
5	Lauryl gallate
6	Ethyl protecatechuate
7	Amyl gallate
8	*n*-Propyl gallate

* B = Brown; Y = yellow.

Results and discussion

The R_F values of the
separation. The minimum a
n-propyl gallate and amyl
stearyl gallate and butyl hy
hydroxytoluene — 4 μg. In
bromine and fluorescein so
spots on a pink background.

The chromatographic
of the polyamide layer chro

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The two spray reagents listed below were sprayed in the order indicated. After spraying the first reagent the chromatogram was dried and observed before spraying with the second reagent.

(1) 5 % (v/v) bromide soln. Dissolve 5 ml of bromine water in 100 ml of carbon tetrachloride.

(2) 0.25 % (w/v) fluorescein sodium soln. Dissolve 250 mg of fluorescein sodium in 5 ml of N,N-dimethylformamide and then add anhydrous ethanol to 100 ml.

TABLE I

CHROMATOGRAPHIC DATA OF PURE COMPOUNDS

System (I) petroleum ether (30–70°)–benzene–acetone (8:2:5); (II) acetone–ethyl alcohol–water (4:1:2).

No.	*Antioxidant*	*R_F values in system*		*Color* of spot at each stage of spraying*	
		I	*II*	*Bromine*	*Fluorescein sodium soln.*
1	Butyl hydroxytoluene	0.98	0.33	B	Y
2	Butyl hydroxyanisole	0.96	0.46	B	Y
3	Stearyl gallate	0.64	0.08	B	Y
4	Cetyl gallate	0.48	0.20	B	Y
5	Lauryl gallate	0.37	0.30	B	Y
6	Ethyl protocatechuate	0.31	0.57	B	Y
7	Amyl gallate	0.17	0.49	B	Y
8	*n*-Propyl gallate	0.09	0.53	B	Y

* B = Brown; Y = yellow.

Results and discussion

The R_F values of the antioxidants are given in Table I and show an excellent separation. The minimum amounts of the compounds detectable by the method are: *n*-propyl gallate and amyl gallate — 0.5 μg; ethyl protocatechuate, lauryl gallate, stearyl gallate and butyl hydroxyanisole — 1 μg; cetyl gallate — 2.5 μg; and butyl hydroxytoluene — 4 μg. In all cases visible spots are obtained after spraying with bromine and fluorescein sodium solutions. This method produces brown or yellow spots on a pink background.

The chromatographic results are highly reproducible. Another important feature of the polyamide layer chromatogram is its capability of direct filing.

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COMMUNICATION

Determination of Isomer Content of Butylated Hydroxyanisole

The isomer content of butylated hydroxyanisole has been determined quantitatively by nuclear magnetic resonance spectroscopy and by gas liquid chromatography. The *tert*-butyl groups of the 2- and 3-*tert*-butyl-4-hydroxyanisole appeared at τ 8.72 and τ 8.63, respectively, in the NMR spectrum. Peak area or peak height was used as a measure of isomer content. Also, gas chromatography of BHA gave two distinct peaks, whose relative areas were used for quantitative determination.

The isomer content of butylated hydroxyanisole (BHA) is important because the antioxidant properties of the two isomers (3-*tert*-butyl-4-hydroxyanisole and 2-*tert*-butyl-4-hydroxyanisole) differ (Whetsel *et al.*, 1957).

The presently available method for the determination of isomer content (Whetsel *et al.*, 1957; Food Chemicals Codex, 1965) is based on quantitative infrared spectroscopy. The method suffers from the disadvantage that relatively large amounts (about 1 gram) of the pure isomers are needed to construct a standard reference curve. Relative isomer content is then calculated from the infrared spectrum of the sample using the reference curve.

The authors now report two simple, fast, and accurate methods which are economical and do not necessarily require reference standard materials.

In the first method, the NMR spectrum of the sample was obtained in carbon tetrachloride. Comparison of the peaks at τ 8.63 and τ 8.72 gave the relative ratios of the 3-isomer of BHA and the 2-isomer, respectively.

In the second method, the sample in chloroform was subjected to gas chromatography. The isomers appeared as two separate peaks, the relative areas of which gave the isomer ratio.

Experimental

Samples. Ten mixtures of the 2- and 3-isomers of BHA containing 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100% by weight of the 2-isomer, respectively, were made from authentic components obtained from Eastman Chemical Products, Inc. (Kingsport, Tenn.). The stated purity was: 3-isomer, 100%; 2-isomer, 98.5%.

Method 1. NMR Spectroscopy. A Varian A60 NMR spectrometer was used. The NMR spectrum of 100 mg. of pure 3-*tert*-butyl-4-hydroxyanisole in 1 ml. of carbon tetrachloride was obtained at 60 Mc. per second in the usual way. The spectrum of pure 2-*tert*-butyl-4-hydroxyanisole was similarly obtained.

The 100-mg. sample of BHA to be analyzed was dissolved in 1 ml. of carbon tetrachloride and its spectrum obtained. The relative isomer content was obtained by measuring the peak heights due to the *tert*-butyl groups at τ 8.72 (2-isomer) and at τ 8.63 (3-isomer). The ratio of these heights gave the isomer ratio. Integrals of these peaks also may be used (Table I).

Method 2. Gas-Liquid Chromatography. The gas chromatograph was an F and M Model 609 with a flame ionization detector.

An aluminum column (7 feet × 1/4 inch) containing

Table I. Comparison of the Results Using NMR and GLC to Determine the Isomer Content of BHA

Sample	% 2-BHA Theoretical	% Found by NMR		% Found by GLC	
		Integral	Peak height	Uncorrected	Corrected
1	0	0	0	0	0
2	10	11.0	11.4	8.1	9.8
3	20	20.4	21.5	16.8	19.9
4	30	29.3	30.8	25.4	29.7
5	40	38.8	40.6	34.4	39.4
6	50	49.0	49.5	44.8	50.0
7	60	59.4	59.5	55.3	60.4
8	70	68.6	68.5	65.9	70.5
9	80	78.0	78.4	76.4	80.0
10	90	88.5	89.1	88.3	90.3
11	100	100	100	100	100

Table II. Positions of NMR Signals of BHA Isomers Relative to Tetramethylsilane (τ 10.0)

Group	NMR Signals 2-Isomer	3-Isomer
$C(CH_3)_3$	8.72	8.63
OCH_3	6.25	6.29
Aromatic H	3.40, 3.29	3.52, 3.21
OH	4.44	5.30

Carbowax 20M (20%) on Chromosorb W (80 to 100 mesh) was used.

The chromatography conditions were as follows. Column temperature 215° C., injection port temperature 280° C., detector temperature 260° C., and attenuation 6400×.

The 100-mg sample was dissolved in 1 ml. of chloroform, and 2 μl. of the solution was injected into the chromatograph. The retention times of the 3-*tert*-butyl-4-hydroxyanisole peak and the corresponding 2-isomer were 25 and 29 minutes, respectively. The peak areas were integrated by triangulation.

Results and Discussion

The 10 samples were analyzed by both methods. Results are presented in Table I.

The positions of the NMR signals due to the *tert*-butyl, methoxyl, and aromatic protons are outlined in Table II. The signals due to *tert*-butyl were sufficiently sharp to allow using the peak height as a quantitative measure.

In the case of the GLC method, the chromatographic response was not the same for each isomer. Comparison of the theoretical and experimental values for each of the 10 mixtures gave an average correction factor of 1.235. (This factor would vary with the instrument.) When this factor was applied to the peak areas of the 2-isomer, the results closely approached theory.

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Separation, Identification and Estimation of Ethyl Gallate, Propyl Gallate, *n*-Octyl Gallate, *n*-Dodecyl Gallate, BHA and BHT by Thin Layer Chromatography

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A rapid and accurate method for the identification and separation of six commonly used antioxidants for fats and oils, namely ethyl gallate, propyl gallate, *n*-octyl gallate, *n*-dodecyl gallate, BHA and BHT, by one-dimensional thin layer chromatography using silica gel G as adsorbent is described. A mixture of equal volumes of ethyl alcohol and acetonitrile has been found to be the best solvent for the extraction of all these antioxidants, and can be used for spotting without any further treatment. As little as 1 µg of BHT and 0·5 µg of gallates and BHA can be detected by this method.

MIXTURES of antioxidants are widely used as preservatives in fats and oils. It has been considered difficult to separate all the antioxidants by one-dimensional chromatography. Seher[1] used two-dimensional thin layer chromatography to separate 12 antioxidants, and Sahasrabudhe[2] separated BHA, BHT, propyl gallate and *n*-octyl gallate by two-dimensional chromatography. Silica gel-kieselguhr mixture was used by Mayer[3] to separate seven antioxidants. Mathew and Mitra used aluminium oxide (GRE Merck) to separate BHA, BHT and propyl gallate[4]. Seher used a mixture of Kiesel gel and 0·5*N* oxalic acid to separate a mixture of antioxidants[5]. The authors have developed a rapid and accurate method for the identification, separation and extraction of the six commonly used antioxidants for fats and oils, namely ethyl gallate, propyl gallate, *n*-octyl gallate, *n*-dodecyl gallate, BHA and BHT by one-dimensional thin layer chromatography using silica gel G as adsorbent.

Complete extraction of all the antioxidants from fats and oils is equally important. Lundberg and Halvorson[6] extracted some of the antioxidants with 80% ethanol except BHT whereas Schwein and Conroy[7] extracted BHT from fat with acetonitrile. The authors have found that a mixture of equal volumes of ethyl alcohol and acetonitrile can extract all the six antioxidants mentioned above and can be used for spotting without any further treatment. As little as 1 µg of BHT and 0·5 µg of gallates and BHA were detected using 20% solution of 2,6-dichloroquinone chlorimide in absolute ethyl alcohol as the chromatogenic agent.

Qualitative determination

TLC plates were prepared by coating glass plates (20×10 cm; TLC kit supplied by M/s *Sinha & Co.*, Calcutta) with a slurry of silica gel G (30 g in 60 ml water) to a thickness of 0·25 mm. The plates were allowed to set for about 30 min, activated in an air oven at 105°C for 1 hr and then stored over silica gel in a desiccator.

Standard solutions of the antioxidants were prepared by dissolving separately 0·005%

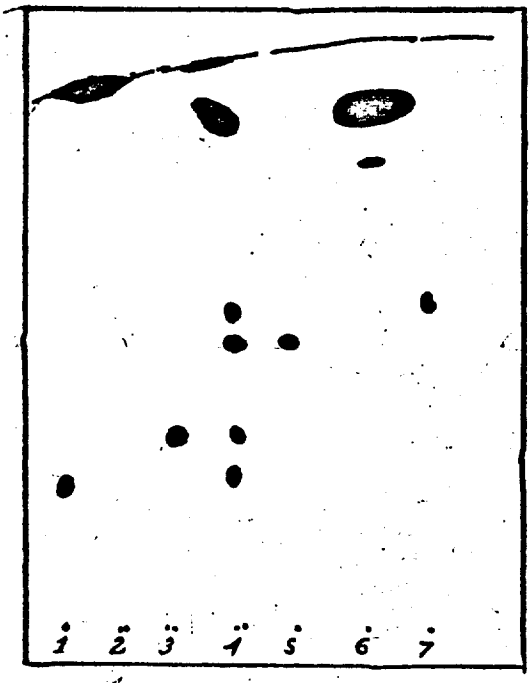

Fig. 1 — Chromatogram showing the separation of antioxidants by TLC using silica gel G as adsorbent [1, ethyl gallate; 2, BHA; 3, propyl gallate; 4, mixture; 5, *n*-dodecyl gallate; 6, BHT; and 7, *n*-octyl gallate]

each of BHA, BHT, propyl gallate, ethyl gallate, *n*-octyl gallate and *n*-dodecyl gallate in double refined antioxidants free groundnut oil. The test sample was prepared by the mixing thoroughly about 40 g groundnut oil (containing unknown antioxidants) with about 20 ml ethanol and 20 ml acetonitrile solution. The mixture was centrifuged and the combined ethanol-acetonitrile layer was taken out. The process was repeated thrice to ensure complete extraction. All the extracts were combined and then concentrated on water-bath to 10 ml. The concentrated solution was used for spotting on the chromatographic plate without any further treatment.

After the initial preparations were over, 10 μl (2 μg) of each antioxidant from the prepared standard solutions and the test sample were spotted in a line 2 cm above the bottom of the plate, the distance between each spot being 1 cm. The plate was

Table 1 — R_f values of the antioxidants

Name of antioxidant	R_f value
Ethyl gallate	0·28
Propyl gallate	0·36
n-Dodecyl gallate	0·58
n-Octyl gallate	0·65
BHA	0·92
BHT	0·99

dipped in a tank containing the mobile solvent, ethylene trichloride-acetic acid-formic acid-isobutanol (15:1:2:2) and the plate was taken out after the solvent front had moved 10 cm from the spotting line. After developing, the plate was kept at room temperature for some time to evaporate the solvent completely and then sprayed with 2% ethanolic solution of 2,6-dichloroquinone chlorimide. Colour development took about 5 min. All the gallates appeared as grey spots, BHT as greyish yellow spot and BHA as orange spot, which changed to violet on exposure to ammonia vapour. The results are presented in Fig. 1. Typical R_f values of all the four gallates, BHA and BHT are given in Table 1.

Quantitative determination

To a sample of double refined antioxidant free groundnut oil, 0·01% solutions of all the six antioxidants, viz. ethyl gallate, propyl gallate, *n*-octyl gallate, *n*-dodecyl gallate, BHA and BHT were mixed well (0·01% solution was prepared by adding 10 mg of each of the antioxidants to 100 ml of groundnut oil). Antioxidants were extracted from this prepared groundnut oil as also from the sample of groundnut oil (100 ml) containing the unknown antioxidants using a mixture of 50 ml alcohol (80%) and 50 ml acetonitrile solution as described earlier. Thin layer plates coated with silica gel G were then spotted with known amounts of antioxidants (1 division of a micropipette of this solution containing 10 μg; and so 1 μg = 1 div.; 2 μg = 2 div.; 3 μg = 3 div.; and so on) and a definite amount (3 μg) of the unknown antioxidants. The plates were developed with the same solvent and under the same conditions as described earlier. After development the plates were

dried properly and only one spot was sprayed with 2,6-dichloroquinone chlorimide solution covering the rest of the plate with a glass plate. Noting the position of these spots, different portions of the plate were scratched out with a sharp edged knife and kept in different test tubes. For example, when the BHA spot is recognized on one corner of the plate by spraying with 2,6-dichloroquinone chlorimide solution, the portions on the same line are obviously considered to be BHA spots.

To each test tube, 20 ml of alcohol was added, shaken vigorously and then filtered. The following treatments were given for different antioxidants.

Treatment for gallates — To the filtrates containing gallates in different test tubes, 2·5 ml of 10% ammonium acetate solution was added. All the test tubes were diluted to 24 ml with distilled water. Then 1 ml of ferrous tartrate reagent (0·1% hydrated ferrous sulphate, $FeSO_4$, $7H_2O$ and 0·5% of Rochelle salt in freshly distilled water) was added to each test tube and mixed well when a pink colour was developed. After 3 min the absorbancy of each solution was read in Klett-Summerson photoelectric colorimeter using blue filter. Graphs were then plotted between the concentration of gallates on X-axis and the colorimeter reading on Y-axis; a straight line was obtained in each case. A typical graph for propyl gallate is given in Fig. 2A. From the graph, the unknown concentration of gallate was found out. The concentrations of all other gallates, namely, ethyl gallate, *n*-dodecyl gallate and *n*-octyl gallate were also estimated by the same method. The method was found sensitive up to 0·5 μg of gallates.

Treatment for BHA — To the filtrates containing BHA in different test tubes, 1 ml of aqueous borax reagent (2% solution of borax $Na_2B_4O_7.10H_2O$ in distilled water) and 0·1% alcoholic solution of 2,6-dichloroquinone chlorimide (2 ml) were added and shaken well when a blue colour was developed. After 10 min, the absorbancy of each solution was found out by Klett-Summerson photoelectric colorimeter using red filter. As above, a graph (Fig. 2B) was plotted and unknown concentration found out. This method is sensitive up to 0·5 μg of BHA in any fat or oil.

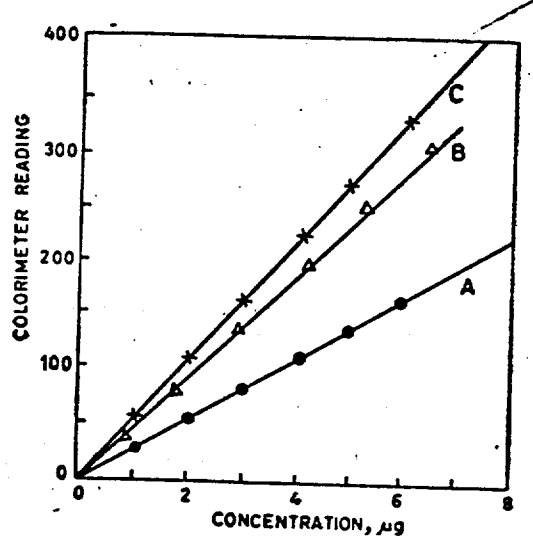

Fig. 2 — Colorimetric reading vs concentration of antioxidant [A, propyl gallate; B, BHT; and C, BHA]

Treatment for BHT — To each test tube containing the BHT extracts, 1 ml of aqueous borax reagent and 0·5% alcoholic solution of phosphomolybdic acid (1 ml) was added and shaken well, when a blue colour was developed. After 10 min the absorbancy of each solution was found out by Klett-Summerson photoelectric colorimeter using red filter as before. A graph (Fig. 2C) was drawn in the same way and unknown amount of BHT found out. The method is sensitive up to 1 μg of BHT in fats and oils.

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Hygienic Evaluation of a Mixture of Butyloxyanisol and Propylgallate Antioxidants of Alimentary Fats

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UDK (Universal Decimal Classification): 613.291 :

(613.268 + 613.288)

The problem of keeping fats from spoiling has great significance for the national economy. In connection with the fact that even at relatively low tem atures there takes place a fat spoilage (for the main part, as a result of oxi tion), the periods for their storage are limited to several months. During ox ation, there is a change not only in the organoleptic indices (taste, color, a odor of the fats), but they also lose valuable constituent parts -- fat-solubl vitamins and highly unsaturated fatty acids, which are subjected to oxidation the first place.

In addition to this, the experimental papers of a number of authors (1, 2, 4) attest to the fact that the toxic action of the oxidation products of fats peroxides,aldehydes, ketones, and polymers. Some papers point out the fact th heated fats, as well as those that have been oxidized, may bring about a devel ment of tumors in the organism (5, 6, 7).

In recent years, in order to prevent fats from being oxidized, there have b proposed chemical compounds that have received the designation of antioxidants antioxidants ((Russian version of the same word -- Translator)). These are ch ical substances that are capable of breaking off the chain reaction of oxidati in rats; they are added in a small amount (0.01 - 0.02%). In investigating th various mixturesof antioxidants, Gearhart and Stuckey (8) came to the conclusi that the most effective one is a mixture of propylgallate and butyloxyanisol; this mixture is 2 times more effective than each component individually. N. Emanuel' and his co-authors (9) have established that the most synergistic eff that these substances yield is in the following concentrations -- 0.01% butyl-

oxyanisol and 0.005% propylgallate.

In the U.S.S.R., for the stabilization of alimentary fats there is permitted application of butyloxytoluol and butyloxyanisol. Propylgallate has been ackno ledged as not being harmless for the organism, and it is not recommended to be applied to nutritive products (10). In our investigations, we made use of this antioxidant in doses 4 times smaller than the doses that manifested a harmful action.

In order to study the toxicity in the mixture of butyloxyanisol and propylga late antioxidants, we carried out acute, subacute and chronic experiments. The purpose of the acute experiments was the determination of the LD_{50} in the mixtu of butyloxyanisol and propylgallate antioxidants, in the ratio of 2 : 1. The purpose of the subacute experiments was the disclosure of the most sensitive te for utilizing them in chronic experiments.

In selecting the tests, we proceeded from the results of the papers (studies carried out in our department, assuming that antioxidants, even in the live organism, act on the oxidizing-reduction processes and on the fat metabolism. acute experiments, which had been carried out on white male rats, having a weig of 100 - 130 grams, it was disclosed that a mixture of butyloxyanisol and propy gallate antioxidants did not manifest any synergistic nor antagonistic action. The LD_{50} (lethal dose) of the mixture of antioxidants, which had been determin by the method of probit (probability unit)-analysis (11), proved to be equal t 2033 plus or minus 140 milligrams/kilogram. It was found to be between the LD for butyloxyanisol (2350 milligrams/kilogram) and that for propylgallate (2300 milligrams/kilogram).

In subacute experiments, a mixture of butyloxyanisol and propylgallate anti oxidants (butyloxyanisol, 100 milligrams/kilogram, and propylgallate, 50 milli grams/kilogram) was administered with a 500-fold aggravation in comparison wit the amount that may find its way into the human organism; this led to a change in the content, in the blood, of ketone bodies, catalase and cholinesterase, a also, of sugar.

In the chronic experiments, which had been conducted on white male rats and on female ones, a clarification was made of the possibility of utilizing a mixture of antioxidants for the stabilization of the alimentary fats.

In the process of investigation, we made a determination of the weight of the animals (in the beginning, after every 10 days, and after that, when the additio (increase) became negligible, after each month), of the activity of the catalase in the blood, in accordance with the procedure of Bakh and Zubkova (12), of the activity of the cholinesterase in the blood, in accordance with the procedure of Khestrin, in the modification of the Kiev Institute of Labor and Trade-Union Diseases, and of the quantitative sugar content in the blood, in accordance with the procedure of Khagedorn (Hagedorn) and Iensen (Jensen)(13). In addition to this, a clarification was made of the content of ketone bodies in the blood and of the albumin (protein) fractions in the blood serum. Daily observations were carried out of the external appearance, behavior of the animals, and of the feed consumption. The experiment was conducted on young, growing animals with a weig of 120 plus or minus 20 grams -- 3 groups of males (1st, 2nd, 3rd) and on groups of females (4th, 5th, 6th), with 10 animals in each one. The animals in the 1st and 4th groups were administered antioxidants (butyloxyanisol and propylgallate) in a 100-fold aggravation (butyloxyanisol, 20 milligrams/kilogram, and propylgallate, 10 milligrams/kilogram) in comparison with the amount that might find way into the human organism; animals of the 2nd and 5th group were administered mixture of antioxidants in a 10-fold aggravation (butyloxyanisol, 2 milligrams/kilogram; propylgallate, 1 milligram/kilogram); the 3rd and 6th group served as control. The antioxidants were given in rendered lard with a feed briquet. Investigations were carried out prior to the beginning of the inoculation and on the 3rd, 6th and 9th months of the inoculation.

In biochemical investigations on the 3rd and 6th month of the experiment, no difference was disclosed between the control and experimental (test) animals. the 6th month, the animals of both groups were joined for the purpose of obtaining a 2nd generation. It turned out that the rats which had received antioxid

ants with their feed were not capable of reproducing progeny: out of 5 animals of the 4th group, which had been subjected to the action of antioxidants, in a 100-fold aggravation, not a single one had any progeny; out of 5 females in the 5th group, which had received antioxidants in a 10-fold aggravation, progeny made their appearance in only one, whereas among the control females, offspring was found in three (see table).

((Table on pg. 49 of text)) below

Results of Experiment on Obtaining Progeny

First Mating					
Antioxidants				Control	
100-fold aggravation		10-fold aggravation			
No. of animal	Amount of offspring	No. of animal	Amount of offspring	No. of animal	Amount of offspring
31	None	41	4	5	Remains of baby rats disclosed
33	"	43	None	53	Same
35	"	45	"	55	None
37	"	47	"	57	"
39	"	49	"	59	7
Second Mating					
32	None	42	2	52	8
34	"	44	None	54	None
36	"	46	"	56	8
38	"	48	"	58	2
40	"	50	"	60	1

((text continues)): For the purpose of verifying the results, the experiment was carried out a second time on other animals. In a secondary mating, out of 5 rats, which had received a mixture of antioxidants in a 100-fold aggravation, not

a single one had any progeny; out of 5 animals to whom antioxidants had been administered in a 10-fold aggravation, only one had any offspring; among the control animals, progeny made its appearance in 4 (see table). A secondary mating confirmed that the animals, which had received antioxidants with their feed, were not capable of reproducing progeny.

On the 9th month of the inoculation, we carried out a sequential series of biochemical investigations. There was disclosed a statistically reliable reduction in the amount of ketone bodies in the blood: P is less than 0.001 in the males, and 0.01 (in the females). Those results were considered as being statistically reliable in which the error probability, P, was less or equal to 0.05 (see illustration). We did not disclose any changes in other biochemical tests. The percent of mortality among the rats that received antioxidants was greater than it was in the control; thus, out of 20 animals to whom had been administered with the feed, antioxidants in a 100-fold aggravation, by the 9th month of the inoculation, 14 remained alive; out of 20 animals that had received antioxidants in a 10-fold aggravation -- 18, in the meantime, in the control groups, out of 20 rats 19 remained alive.

Thus, a mixture of antioxidants consisting of butyloxyanisol and propylgallate proved to be toxic for rats, and in connection with this, should not be permitted for the stabilization of alimentary fats.

Conclusions

1. A mixture of antioxidants, consisting of butyloxyanisol and propylgallate, in a 100-fold aggravation, in the case of its prolonged feeding to rats, increases the mortality of the experimental (test) animals in comparison with the control (ones).

2. The administration, to the experimental (test) animals, of the indicated mixture of antioxidants, brings about sterility in the animals.

3. Butyloxyanisol and propylgallate, in their action on the organism, lead to a reduction in the amount of ketone bodies in the blood. Changes in the sugar content, and also, in the activity of enzymes (catalase and cholinesterase) in

the blood, were not observed.

4. A mixture of butyloxyanisol and propylgallate antioxidants appears to be toxic for the organism and cannot be employed for the stabilization of alimentary fats.

((Double illustration on pg. 50 of text))((to the left of both parts of the illustration)): Amount of ketone bodies in the blood (in % milligrams); ((within the same illustration, at the top of each part)): Males; Females: ((immediately underneath the lower part of this illustration)): Prior to the experiment; 3rd month; 6th month; 9th month; Time of observation; ((below that)): Change in the content of ketone bodies in the blood of experimental (test) and control rats.

Designations: a -- animals that had received antioxidants in a 100-fold aggravation; ((Russian)) b -- animals that had received antioxidants in a 10-fold aggravation; ((Russian)) v -- control (having received no antioxidants). The numbers inside the columns designate the average (mean) magnitudes of the results obtained. P represents the probability of error.

((within the upper part of the abovementioned illustration, at the lower left)): a; ((Russian)) b; ((Russian)) v.

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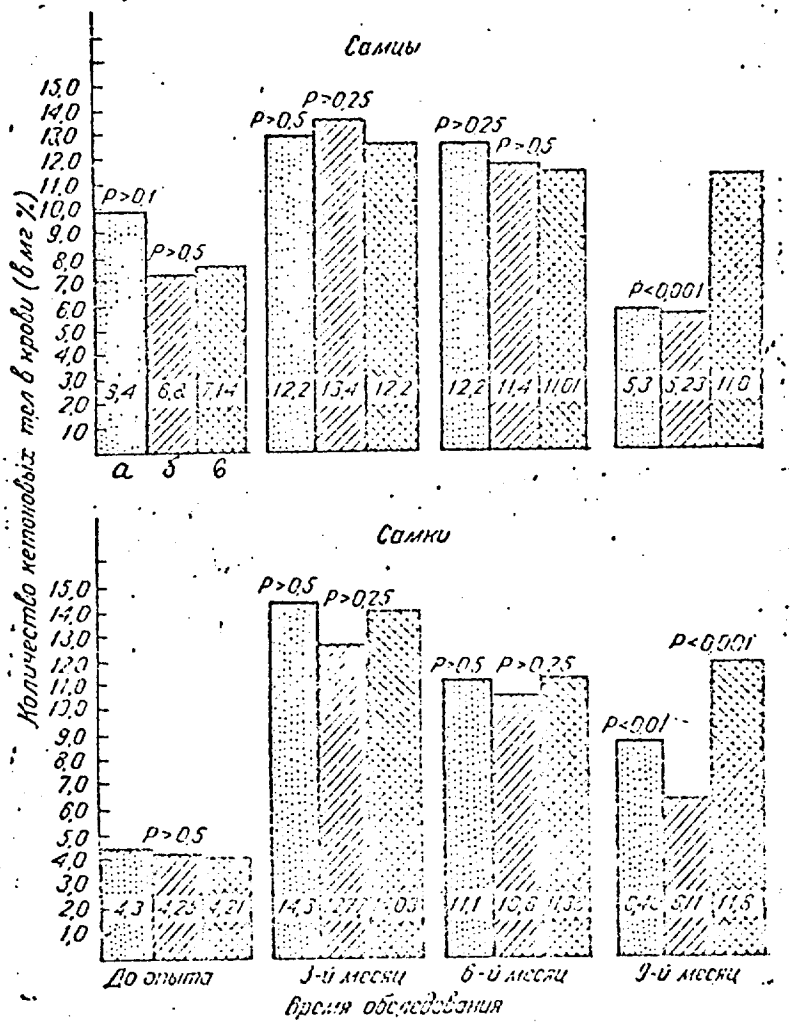
Самцы
Самки
Количество кетоновых тел в крови (в мг %)
P>0,1
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Поступила 1/XI 1965 г.

EFFECTS PRODUCED BY COTTON SEED OIL AND MUTTON FAT, SUBJECTED TO HEAT TREATMENT, ON THE LEVEL OF CERTAIN FATTY ACIDS IN THE BLOOD AND LIVER OF EXPERIMENTAL ANIMALS

N. G. Popova, L. R. Losieva, M. N. Ismailov (Tashkent)

Summary

Protracted experiments were conducted on growing albino male rats to study the action of qualitatively different fats upon the polyunsaturated fatty acids content in the blood serum and the liver. As distinct from the mutton fat the cotton seed oil was found to raise the level of polyunsaturated fatty acids in the blood serum of rats. The liver of rats receiving cotton seed oil contained 2.5—5 times as much of unsaturated fatty acids as that of rats kept on a mutton-fat diet. Feeding on a heated cotton seed oil results in the reduced level of polyunsaturated fatty acids in the blood serum and in the rise of their content in the liver of rats (exept for linolenic acid).

УДК 613.291 : [613.268+613.288]

ГИГИЕНИЧЕСКАЯ ОЦЕНКА СМЕСИ АНТИОКИСЛИТЕЛЕЙ ПИЩЕВЫХ ЖИРОВ БУТИЛОКСИАНИЗОЛА И ПРОПИЛГАЛЛАТА

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Проблема предохранения жиров от порчи имеет большое народнохозяйственное значение. В связи с тем, что даже при относительно низких температурах происходит порча жиров (главным образом в результате окисления), сроки их хранения ограничиваются несколькими месяцами. При окислении изменяются не только органолептические показатели (вкус, цвет, запах жиров), но они утрачивают и ценные составные части — жирорастворимые витамины и высоконепредельные жирные кислоты, подвергающиеся окислению в первую очередь.

Помимо того, экспериментальные работы ряда авторов [1, 2, 3, 4] свидетельствуют о токсическом действии продуктов окисления жиров — перекисей, альдегидов, кетонов, полимеров. Некоторые работы говорят о том, что гретые жиры, так же как и окисленные, могут вызывать развитие опухолей в организме [5, 6, 7].

В последние годы для предохранения жиров от окисления предложены химические соединения, получившие название антиоксидантов или

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антиокислителей. Это — химические вещества, способные прерывать цепную реакцию окисления в жирах; добавляются они в небольшом количестве (0,01—0,02%).

Исследуя различные смеси антиокислителей, gearhart и stuckey [8] пришли к заключению, что наиболее эффективной является смесь пропилгаллата и бутилоксианизола; эта смесь в 2 раза эффективнее, чем каждый компонент в отдельности. П. Эмануэль с соавторами [9] установили, что наибольший синергический эффект эти вещества дают в концентрациях: бутилоксианизол — 0,01% и пропилгаллат — 0,005%.

В СССР для стабилизации пищевых жиров разрешено применение бутилокситолуола и бутилоксианизола. Пропилгаллат признан небезвредным для организма и к применению в продуктах питания не рекомендован [10]. Мы в исследованиях применяли этот антиокислитель в дозах в 4 раза меньших, чем дозы, оказывавшие вредное действие.

Для изучения токсичности смеси антиокислителей бутилоксианизола и пропилгаллата нами были проведены острый, подострый и хронический эксперименты.

Целью острых опытов являлось определение $ЛД_{50}$ смеси антиокислителей бутилоксианизола и пропилгаллата в соотношении 2 : 1. Целью подострых опытов было выявление наиболее чувствительных тестов для использования их в хронических опытах.

При выборе тестов мы исходили из результатов работ, проводимых на нашей кафедре, допуска, что антиокислители и в живом организме действуют на окислительно-восстановительные процессы и жировой обмен.

В острых опытах, проводившихся на белых крысах-самцах весом 100—130 *г*, обнаружено, что смесь антиокислителей бутилоксианизола и пропилгаллата не проявляла ни синергического, ни антагонического действия. $ЛД_{50}$ смеси антиокислителей, определявшаяся методом пробит-анализа [11], оказалась равной 2633±140 *мг/кг*. Она находилась между $ЛД_{50}$ бутилоксианизола (2950 *мг/кг*) и пропилгаллата (2500 *мг/кг*).

В подострых опытах смесь антиокислителей бутилоксианизола и пропилгаллата (бутилоксианизола 100 *мг/кг*, пропилгаллата 50 *мг/кг*) вводили с аггравацией в 500 раз по сравнению с количеством, которое может попасть в организм человека, что приводило к изменению содержания в крови кетоновых тел, каталазы и холинэстеразы, а также сахара.

В хронических опытах, проводившихся на белых крысах-самцах и самках, выясняли возможность использования смеси антиокислителей для стабилизации пищевых жиров.

В процессе исследования мы определяли вес животных (вначале через каждые 10 дней, а затем, когда прибавление стало незначительным, через каждый месяц), активность каталазы в крови по методике Баха и Зубковой (12), активность холинэстеразы в крови по методике Хестрина в модификации Киевского института труда и профзаболеваний, количественное содержание сахара в крови по методу Хагедорна и Иенсена [13]. Кроме того, выясняли содержание кетоновых тел в крови и белковых фракций в сыворотке крови. Ежедневно проводили наблюдения за внешним видом, поведением животных и поедаемостью корма. Опыт ставили на молодых, растущих животных весом 120±20 *г* — 3 группах самцов (1-я, 2-я, 3-я) и 3 группах самок (4-я, 5-я и 6-я) по 10 животных в каждой. Животным 1-й и 4-й группы антиокислители (бутилоксианизол и пропилгаллат) давали в 100-кратной аггравации (бутилоксианизола 20 *мг/кг*, пропилгаллата 10 *мг/кг*) по сравнению с количеством, которое может попасть в организм человека; животным 2-й и 5-й группы смесь антиокислителей вводили в 10-кратной аггравации (бутилоксианизола 2 *мг/кг*; пропилгаллата 1 *мг/кг*); 3-я и 6-я группа служила контролем. Антиокислители давали в свином топленом жире с кормовым брикетом. Исследования проводили до начала затравки и на 3, 6 и 9-м месяце затравки.

При биохимических исследованиях на 3-м и 6-м месяце эксперимента разницы между контрольными и подопытными животными не выявлено.

as urged to investigate its copyright status.

На 6-м месяце животных обеих групп соединили для получения 2-го поколения. Оказалось, что крысы, получавшие с кормом антиокислители, к воспроизведению потомства не способны: из 5 животных 4-й группы, подвергавшихся воздействию антиокислителей в 100-кратной аггравации, ни у одного не было потомства; из 5 самок 5-й группы, получавших антиокислители в 10-кратной аггравации, потомство появилось только у одной, тогда как среди контрольных самок приплод был у трех (см. таблицу).

Результаты эксперимента по получению потомства

Первая подсадка					
антиокислители				контроль	
100-кратная аггравация		10-кратная аггравация			
№ животного	количество приплода	№ животного	количество приплода	№ животного	количество приплода
31	Нет	41	4	51	Обнаружены остатки крысят
33	»	43	Нет	53	То же
35	»	45	»	55	Нет
37	»	47	»	57	»
39	»	49	»	59	7
Вторая подсадка					
32	Нет	42	2	52	8
34	»	44	Нет	54	Нет
36	»	46	»	56	8
38	»	48	»	58	2
40	»	50	»	60	1

Для подтверждения результатов эксперимент был проведен повторно на других животных. При повторной подсадке из 5 крыс, получавших смесь антиокислителей в 100-кратной аггравации, ни одна не имела потомства; из 5 животных, которым антиокислители вводили в 10-кратной аггравации, приплод был только у одной; среди контрольных животных потомство появилось у 4 (см. таблицу). Повторная подсадка подтвердила, что животные, получавшие с кормом антиокислители, к воспроизведению потомства не способны.

На 9-м месяце затравки нами был проведен очередной цикл биохимических исследований. Выявлено статистически достоверное уменьшение количества кетоновых тел в крови: $P<0{,}001$ у самцов и 0,01 (у самок). Статистически достоверными считали результаты, в которых вероятность ошибки P была меньше или равнялась 0,05 (см. рисунок). Изменений других биохимических тестов нами не выявлено.

Процент гибели среди крыс, получавших антиокислители, был выше, чем в контроле; так, из 20 животных, которым вводили с кормом антиокислители в 100-кратной аггравации, к 9-му месяцу затравки осталось в живых 14; из 20 животных, получавших антиокислители в 10-кратной аггравации, — 18; между тем в контрольных группах из 20 крыс осталось в живых 19.

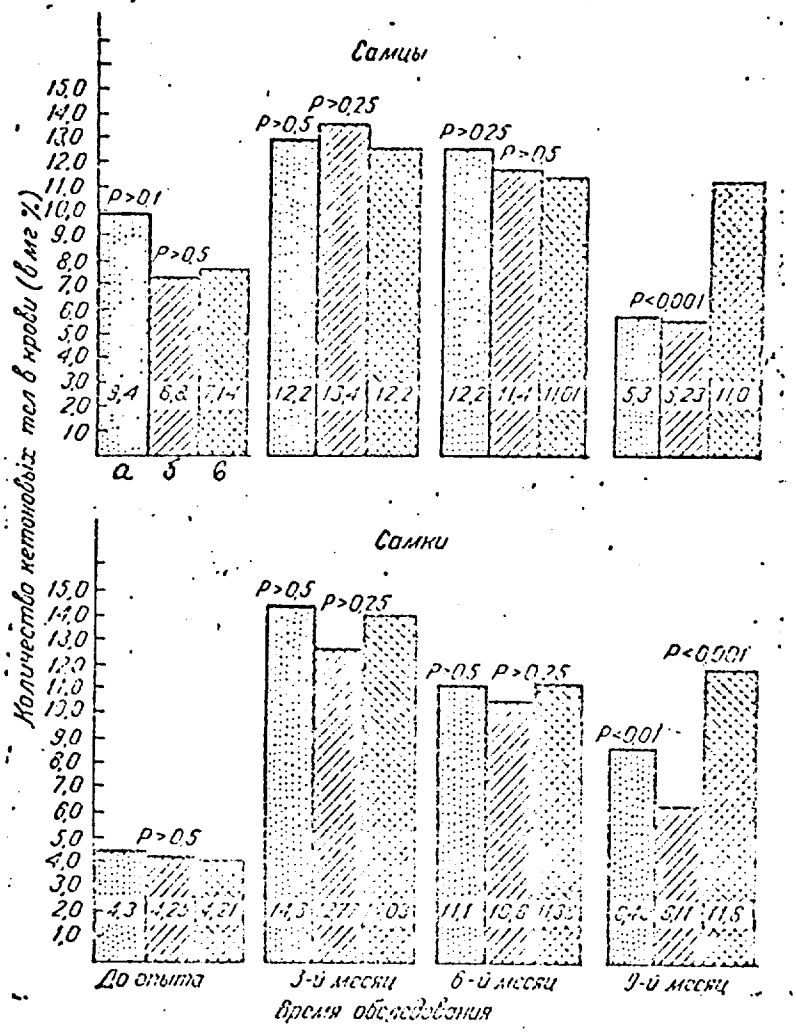
Таким образом, смесь антиокислителей бутилоксианизола и пропилгаллата оказалась токсичной для крыс и в связи с этим не должна быть допущена для стабилизации пищевых жиров.

Выводы

1. Смесь антиокислителей бутилоксианизола и пропилгаллата в 100-кратной аггравации при длительном скармливании ее крысам увеличивает смертность подопытных животных по сравнению с контролем.

2. Введение подопытным животным указанной смеси антиокислителей вызывает у животных бесплодие.

3. Бутилоксианизол и пропилгаллат при воздействии на организм крыс приводят к снижению количества кетоновых тел в крови. Измене-



Изменение содержания кетоновых тел в крови подопытных и контрольных крыс.

Обозначения: *а* — животные, получавшие антиокислители в 100-кратной аггравации; *б* — животные, получавшие антиокислители в 10-кратной аггравации; *в* — контроль (не получавшие антиокислителей). Цифры внутри столбцов обозначают средние величины полученных результатов. *P* — вероятность ошибки.

ний в содержании сахара, а также активности ферментов (каталаза и холинэстераза) в крови не наблюдалось.

4. Смесь антиокислителей бутилоксианизола и пропилгаллата является токсичной для организма и не может применяться для стабилизации пищевых жиров.

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HYGIENIC ASSESSMENT OF A MIXTURE CONTAINING ANTIOXIDIZERS OF ALIMENTARY FATS — BUTYLOXYANISOLE AND PROPYL GALLATE

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Summary

A mixture comprising butyloxyanisole and propyl gallate — antioxidizers of alimentary fats — was studied for its effect on the organism after long-term administration. Experiments were carried out on albino rats. The mixture of antioxydizers caused a greater destruction of animals, while the surviving ones lost the ability to reproduce offsprings.

The obtained data point to the toxic action of the discussed mixture and to the impossibility of employing it as an antioxidizer for alimentary fats.

УДК 613.268 : 613.295

ИССЛЕДОВАНИЕ СТОЙКОСТИ НЕКОТОРЫХ РАСТИТЕЛЬНЫХ ЖИРОВ, ИСПОЛЬЗУЕМЫХ ДЛЯ ФРИТЮРНОЙ ЖАРКИ

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В общественном питании и торговле ежегодно реализуется не менее 2 млрд. порций кулинарных изделий, приготавливаемых обжариванием во фритюре. Сюда относятся пирожки жареные, пончики, гарнирный картофель, хрустящий картофель, рыба, овощи и другие продукты. Они находят широкое распространение ввиду их высоких вкусовых достоинств и питательности.

Для удовлетворения растущего спроса на эти изделия организовано централизованное поточно-механизированное производство некоторых из них на предприятиях промышленного типа. Техника и технология производства обжаренных во фритюре продуктов быстро развиваются на основе применения полуфабрикатов. Однако в процессе длительного и многократного использования фритюрных жиров последние претерпевают физико-химические превращения [1—4], в результате которых их пищевая и биологическая ценность существенно уменьшается или даже полностью исчезает [5]. Кроме того, в таких жирах возможно образование веществ, не безвредных для здоровья человека [2].

Эти обстоятельства приводят к образованию больших отходов (неиспользуемых в пище остатков) фритюрных жиров, что удорожает кулинарное производство и сдерживает его развитие. Вот почему подбор наиболее термостойких жиров или их смесей, а также оптимальной технологии использования этих продуктов в качестве фритюра является важной экономической и гигиенической задачей.

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The Pharmacological Evaluation of Antioxidants

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I. Introduction

Investigations into the problem of rancidity and methods for stabilizing edible oils and fats have been quite extensive. One of the fundamental problems yet to be solved is the mechanism by which fats become rancid and the role of antioxidants in the prevention or delay of rancidification. Mattill (1947) has reviewed the chemical aspects of the problem. The influence of lipoxidases, yeasts, molds, or bacteria on the decomposition of glycerides of fatty acids has also not been clearly elucidated although it is known that the presence of such entities is not necessary for the production of rancidity.

The importance of preserving the wholesomeness of edible fats is exemplified by the fact that in the United States the average individual derives about one-third of his total caloric requirements from fat. Fat not only functions as a concentrated foodstuff, but also has an important relationship to the availability and utilization of essential dietary substances. Some of these substances are vitamin A, carotene, vitamin E, vitamin D, pantothenic acid, pyridoxine, biotin, ascorbic acid, and linoleic acid (Burr and Barnes, 1943). Considerable experimental evidence is available also which emphasizes that the diet of man and animals is enhanced in some way by the addition of fresh natural edible fats. The presence of rancid fats in the diet, however, has a serious deleterious

effect, which goes beyond the destruction of essential dietary constituents. Fitzhugh *et al.* (1944) have demonstrated that the feeding of rancid lard to rats at a dietary level of 6% resulted in a marked emaciation of the animals by the end of the first year on the diet. A progressive type of paralysis was also noted in these animals at the time of termination of the experiments (2 years). The pathological examination verified the fact that the major symptoms were the result of a vitamin E deficiency, although the byproducts of the oxidized fat appear to have a toxic action of their own (Burr and Barnes, 1943).

From these considerations the indications are that considerable emphasis must be placed on the importance of keeping edible fats palatable and wholesome, especially when storage is necessary. Stabilization of fats has been accomplished by several means, such as choice of raw materials, hydrogenation, proper packaging, deodorization, and exercising every precaution during processing to avoid unnecessary exposure to heat and light, or contamination with prooxidants. All these methods have their limitations in that their applications are not always feasible to all fats and fatty food products; hence reliance for the stabilization of fats has been placed on antioxidants. By the addition of traces of certain chemicals it has been found that oxidative rancidity can be inhibited and the age resistance of fats considerably increased. This phenomenon has been known for more than 100 years and the first utilization for prevention of rancidity was in the pharmaceutical field—employment of gum benzoin to protect lard. This product has been official in the United States Pharmacopoeia since 1880.

Many compounds have been found to possess antioxidant properties, the more common compounds being gum guaiac, gallic acid, catechol, and hydroquinone. Numerous other chemicals of the phenolic and acidic type have also had their proponents. Naturally occurring antioxidants have received considerable attention, the purpose being to develop a nontoxic substance suitable for food use. Lecithin, cephalin, tocopherols, and products obtained from cereals, yeasts, sugars, and other food substances have been proposed. Lundberg (1947) has compiled a list of antioxidants which are in current use or which have been proposed for use in stabilizing edible fats.

The criteria which are considered necessary for establishing the usefulness of a chemical as an antioxidant have been listed by Higgins and Black (1944). According to these authors, the antioxidant should be soluble in fats and impart no foreign color, odor, or flavor to the fat even on long storage and should be an effective inhibitor in the treated product for at least a year after storage at a temperature of 75°–85° F. in unsealed containers. The antioxidant should be unchanged when

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heated and should possess the ability of retarding rancidity in baked foods prepared from fats treated with the antioxidant. The safety of the antioxidant for food use must be established. There are many antioxidants which are able to pass the physical and chemical criteria for practical usefulness, but to establish proof of nontoxicity is more difficult.

Antioxidants are chemical preservatives and come under the terms of the Federal Food, Drug and Cosmetic Act when they are present in food shipped in interstate commerce. Any antioxidant which may be legally incorporated into foods must be declared on the label in a way to show it to be a preservative. If the chemical is safe and nonpoisonous, it can be incorporated into unstandardized foods as a preservative if it does not conceal damage or inferiority or make the product appear better or of greater value. It may be used in standardized food only if the standard recognizes the preservative as a legitimate ingredient. If the food is not a standardized item and the antioxidant is nonpoisonous, the only requirement is the declaration of the preservative on the label. If the antioxidant is a poisonous and deleterious substance it must be shown that the preservative is required and cannot be avoided in good manufacturing practice. In this event, the law provides that the Administration shall promulgate regulations limiting the quantity of the substance a food may contain so that it will not constitute a hazard to the public health. It is the manufacturer's and shipper's responsibility to secure adequate evidence of safety for the antioxidant that he intends to use. A general plan for the pharmacological investigation, which permits a thorough appraisal of the toxicology of an antioxidant, has been presented (Lehman, 1948).

Of the many chemicals proposed for antioxidant use and under active investigation, pharmacological data on the compounds listed below have become available.

1. Nordihydroguaiaretic acid
2. Propyl gallate
3. Hydroquinone
4. l-ascorbyl palmitate
5. *d*-isoascorbyl palmitate
6. *d*-isoascorbic acid
7. Thiodipropionic acid
8. Dilauryl thiodipropionate
9. Distearyl thiodipropionate
10. Gum guaiac

The palmitates and *d*-isoascorbic acid are not considered very effective as antioxidants and any discussion of their pharmacology will be incidental only. Phenol and catechol are included for the purpose of comparison, although they are too toxic for food use.

II. Acute Toxicity

A summary of the acute toxicity of the antioxidants is presented in Table I. From a practical standpoint it may be stated that an antioxidant which possesses an LD_{50} (the quantity of the antioxidant which is fatal to 50% of the test animals following oral administration) of 1,000 mg./kg. or above has met at least one important criterion of harmlessness. An inspection of the table reveals that nordihydroguaiaretic acid, propyl gallate, thiodipropionic acid and its derivatives, *d*-isoascorbyl palmitate, gum guaiac, and butylated hydroxy anisole have measured up to this criterion. It is of interest to note that in general hydroquinone and phenol have the same order of toxicity.

Table I

The Acute Toxicity of Antioxidants

Animal	Route of administration	Approximate LD_{50} mg./kg.	Source
		Nordihydroguaiaretic acid	
Mice	Oral	>2000	Bieter (1949)
Mice	Oral	4000	F & D A* (1950)
Rats	Oral	>2000	Bieter (1949)
Rats	Oral	5500	F & D A* (1950)
Guinea pigs	Oral	830	Bieter (1949)
Mice	Intraperitoneal	550	Bieter (1949)
		Propyl gallate	
Mice	Oral	2000	Boehm and Williams (1943)
Mice	Oral	3500	F & D A* (1950)
Rats	Oral	3800	Orten *et al.* (1948)
Rats	Oral	3600	F & D A* (1950)
Rats	Intraperitoneal	380	Orten *et al.* (1948)
		Thiodipropionic acid	
Mice	Oral	>2000	Hazleton (1949)
Mice	Oral	2000	F & D A* (1950)
Mice	Intraperitoneal	250	Hazleton (1949)
Mice	Intravenous	175	Hazleton (1949)
Rats	Oral	>2000	Hazleton (1949)
Rats	Oral	3000	F & D A* (1950)
Rats	Intraperitoneal	500	Hazleton (1949)
Rats	Intravenous	>300	Hazleton (1949)
		Dilauryl thiodipropionate	
Mice	Oral	>2000	Hazleton (1949)
Rats	Oral	>2500	Hazleton (1949)
Mice	Intraperitoneal	>2000	Hazleton (1949)

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	Source
ic acid	
	ter (1949)
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	Bieter (1949)
	F & D A* (1950)
	Bieter (1949)
	Bieter (1949)
e	
	Boehm and Williams (1943)
	F & D A* (1950)
	Orten *et al.* (1948)
	F & D A* (1950)
	Orten *et al.* (1948)
acid	
	Hazleton (1949)
	F & D A* (1950)
	Hazleton (1949)
	Hazleton (1949)
	Hazleton (1949)
	F & D A* (1950)
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pionate	
	Hazleton (1949)
	Hazleton (1949)
	Hazleton (1949)

TABLE I (*Continued*)

Animal	Route of administration	Approximate LD_{50} mg./kg.	Source
		Distearyl thiodipropionate	
Mice	Oral	>2000	Hazleton (1949)
Rats	Oral	>2500	Hazleton (1949)
Mice	Intraperitoneal	>2000	Hazleton (1949)
		d-Isoascorbyl palmitate	
Mice	Oral	>8000	F & D A* (1950)
Rats	Oral	6000	F & D A* (1950)
		Gum guaiac	
Mice	Oral	>2000	Bieter (1949)
Rats	Oral	>2000	Bieter (1949)
Rats	Oral	>5000	F & D A* (1950)
Guinea pigs	Oral	1120	Bieter (1949)
Mice	Intraperitoneal	>2000	Bieter (1949)
		Catechol	
Mice	Oral	260	Bieter (1949)
Rats	Oral	260	Bieter (1949)
Guinea pigs	Oral	210	Bieter (1949)
Mice	Intraperitoneal	190	Bieter (1949)
		Butylated hydroxy anisole	
Mice	Oral	2000	F & D A* (1950)
Rats	Oral	2200	F & D A* (1950)
		Hydroquinone	
Mice	Oral	400	F & D A* (1950)
Rats	Oral	320	F & D A* (1950)
Guinea pigs	Oral	550	F & D A* (1950)
Pigeons	Oral	300	F & D A* (1950)
Dogs	Oral	200	F & D A* (1950)
Cats	Oral	70	F & D A* (1950)
Rats	Intravenous	115	F & D A* (1950)
		Phenol	
Mice	Oral	520	Bieter (1949)
Mice	Oral	395	F & D A* (1950)
Rats	Oral	440	Bieter (1949)
Rats	Oral	450	F & D A* (1950)
Mice	Intraperitoneal	360	Bieter (1949)

* Food and Drug Administration, unpublished data.

III. Chronic Toxicity

1. Rats

Lifetime feeding studies in rats have been carried out with all the compounds listed. One of the criteria for establishing the harmlessness of a substance which may be ingested chronically when added to food is the effect of such a substance on the growth rate of rats. The first six

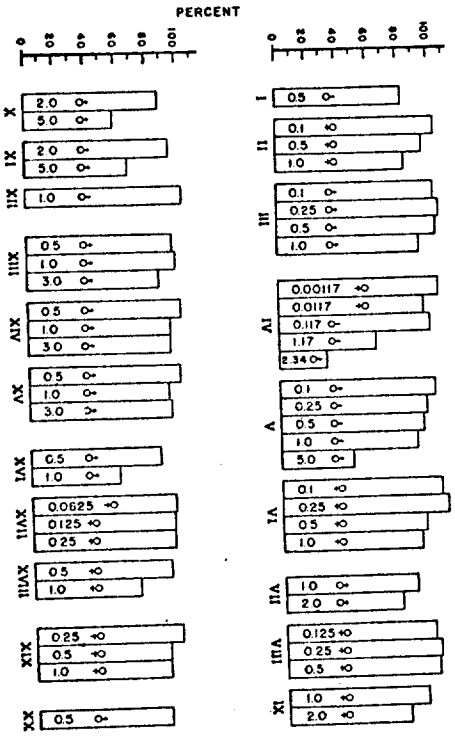

Fig. 1. Mean growth rate of rats fed the antioxidants for 6 months expressed as per cent of the control groups. The growth rate of the control animals was taken as 100 per cent. The figures in each column represent the per cent of the antioxidant in the diet. The symbol ♂ = male; ♀ = female. The Roman numerals identify the antioxidants and source of the data as follows: I, II, Nordihydroguaiaretic acid (Bieter); III, Nordihydroguaiaretic acid (F&DA*); IV, Propyl gallate, (Orten *et al.*); V and VI, Propyl gallate, (F&DA*); VII, VIII, IX, Hydroquinone, (F&DA*); X, *l*-ascorbyl palmitate, (F&DA*); XI, *d*-isoascorbyl palmitate, (F&DA*); XII, *d*-isoascorbic acid, (F&DA*); XIII, Thiodipropionic acid, (Hazleton); XIV, Dilauryl thiodipropionate, (Hazleton); XV, Distearyl thiodipropionate, (Hazleton); XVI, XVII, XVIII, Catechol, (F&DA*); XIX, Phenol, (F&DA*); XX, Gum guaiac, (Bieter).

* Food and Drug Administration, unpublished data.

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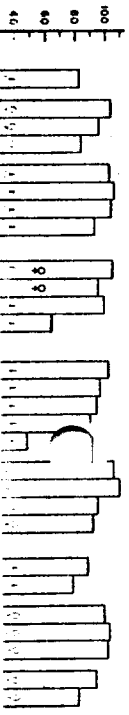

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: IV, Propyl gallate, (Orten *et*
I, IX, Hydroquinone, (F&DA*);
yl palmitate, (F&DA*); XII,
cid, (Hazleton); XIV, Dilauryl
propionate, (Hazleton); XVI,
(F&DA*); XX, Gum guaiac,

months in the life of a rat is usually taken for observing growth changes for the reason that this time period represents the rapidly growing stage. Figure 1 represents a summary of the data of the various compounds. Practically all of the antioxidants are recommended for use in quantities not to exceed 0.01% in animal fats and shortenings. In assessing the harmlessness of the antioxidants, a factor of safety of at least 100 is taken. This requires that when the antioxidant is fed to experimental animals in their total diet at a level 100 times that proposed in fats for human consumption, no significant effect on growth should occur. The results in Fig. 1 are unfavorable to nordihydroguaiaretic acid and hydroquinone. Orten *et al.* (1948) noted some stunting in rats on the 1.17% level of feeding propyl gallate. No significant effect at the 1% level was noted in our rats, but retardation was significant at the 5% level. It may be noted in the figure that we have fed all the antioxidants for a lifetime with the exception of thiodipropionic acid and its esters and gum guaiac. This affords an opportunity to compare the growth effects of the various antioxidants on an equal basis. Since it required 5% of propyl gallate to affect growth in our experiments, it is believed that species difference or some other variable was responsible for the stunting of growth in the 1.17% level of the antioxidant as observed by Orten *et al.*

The dilauryl and distearyl esters of thiodipropionic acid are recommended for use in a concentration of 0.09%. These compounds represent about 0.03% of the acid and since they have been fed at a level of 3.0% without evidence of effect, the 100-times margin of safety appears to have been established.

Gum guaiac is employed in concentrations of 0.1%, and to meet the criterion about a 10% feeding level should have been carried out. Very often technical difficulties do not permit the addition of such high concentrations as would be necessary in the case of gum guaiac to establish a 100-fold margin of safety. This obstacle can be overcome in many instances by supplementary data in other species of animals. This has not been done with gum guaiac, but the long history of its use in medicine without reported injury appears to substantiate the harmlessness of the gum for antioxidant use.

2. *Other Species*

Chronic studies on propyl gallate have been conducted in guinea pigs and dogs by Orten *et al.* (1948). The guinea pigs were fed at a level of 0.011% of the antioxidant and were observed for one year. No detectable effect on the rate of growth was noted. No influence on reproduction was noted.

A group of 7 dogs was fed propyl gallate for 14 months in amounts

similar to that fed the guinea pigs. The animals on the propyl gallate regimen showed no demonstrable effects and were similar in every respect to the 5 animals which served as controls.

Thiodipropionic acid was fed to guinea pigs by Hazleton (1949) at a level of 0.5% in the drinking water and for a period of 120 days. No significant effect was noted on weight or mortality. Dogs were fed a mixture of 10 parts by weight of dilauryl thiodipropionate and one part of thiodipropionic acid and in a concentration of 0.1% and 3.0% of the acid-ester mixture in the diet. The observation period was 100 days. No untoward effects were noted.

Nordihydroguaiaretic acid has not been subjected to additional studies in dogs which is a serious omission in light of the results in rats. Bieter (1949) employed mice in one series of feeding experiments but the results were inconclusive.

Some observations on the blood constitutents were made by Orten *et al.* (1948) on rats receiving propyl gallate in the diet. The lower levels of antioxidant feeding produced no changes, but concentrations above 1.1% produced significantly lower hemoglobin values. Erythrocite and leucocyte counts were not significantly affected. Hazleton (1949) made similar blood studies on the dogs fed the acid-ester mixture of thiodipropionic acid without finding anything of significance.

In the pharmacological appraisal of an antioxidant it is important to determine the effect of heat on the stability of the substance. Reaction products or a modification of the chemical may occur which may have pharmacological effects. Orten *et al.* (1948) included such experiments in their observations. Rats were fed a heated antioxidant mixture (propyl gallate, lecithin, and corn oil) in an amount at least 100 times that which would be consumed by humans if all the dietary fat were treated with the recommended amount of antioxidant mixture effective in preserving edible fat. No detectable toxic effects were noted. Hazleton (1949) also fed thiodipropionic acid in combination with dilauryl thiodipropionate heated with lard in concentrations at least 10 times that recommended for use as an antioxidant. No injury was noted. We have conducted similar experiments with the ascorbyl palmitates without evidence of injury.

3. Mortality

The mortality rate of rats fed the several antioxidants is also an index of toxicity. A high mortality in any group of treated animals as compared with the controls indicates that the substance under test is quite toxic. If the mortality rate is about equal to that of the controls, the compound may be considered as not highly toxic. Table II summarizes

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the available data on the mortality rates of the various compounds. It may be seen that none of the compounds affected the mortality rate of the rats. None of the treated groups showed significant differences from the controls in the number of animals living at any time up to the end of the experiment.

TABLE II

Mortality in Rats after 2 Years of Feeding the Several Antioxidant Diets

Antioxidant	Dosage per cent	Number of Animals Per group	Number of Animals Living at 2 years	Source
Nordihydroguaiaretic acid	0	18	13	Bieter (1949)
	0.1	18	12	
	0.5	18	17	
	1.0	18	12	
Nordihydroguaiaretic acid	0	10	3	F & D A* (1950)
	0.1	10	1	
	0.25	10	3	
	0.5	10	3	
	1.0	10	2	
Propyl gallate	0	16	8	F & D A* (1950)
	0.1	16	4	
	0.25	16	6	
	0.5	16	6	
	1.0	16	4	
	5.0	16	3	
Thiodipropionic acid	0	20	17	Hazleton (1949)
	0.5	20	16	
	1.0	20	13	
	3.0	20	15	
Dilauryl thiodipropionate	0.5	20	10	Hazleton (1949)
	1.0	20	13	
	3.0	20	4	
Distearyl thiodipropionate	0.5	20	18	Hazleton (1949)
	1.0	20	14	
	3.0	20	18	
l-ascorbyl palmitate	0	10	4	F & D A * (1950)
	0.05	10	6	
	0.25	10	4	
d-isoascorbyl palmitate	0.05	10	4	F & D A * (1950)
	0.25	10	5	
d-isoascorbic acid	1.0	10	4	F & D A * (1950)
Gum guaiac	0	10	7	Bieter (1949)
	0.5	10	9	

TABLE II (*Continued*)

Antioxidant	Dosage per cent	Number of Animals Per group	Living at 2 years	Source
Catechol	0	12	2	F & D A* (1950)
	0.0625	12	4	
	0.125	12	4	
	0.25	12	1	
Catechol	0	18	5	F & D A* (1950)
	0.5	18	4	
	1.0	18	4	
Hydroquinone	0	12	2	F & D A* (1950)
	0.125	12	5	
	0.25	12	8	
	0.5	12	8	
Hydroquinone	0	18	5	F & D A* (1950)
	1.0	18	7	
	2.0	18	5	
Phenol	0	12	2	F & D A* (1950)
	0.25	12	5	
	0.5	12	6	
	1.0	12	6	

* Food and Drug Administration, unpublished data.

IV. PATHOLOGY

The predominant pathologic lesions produced by the various antioxidants after lifetime feeding to rats are summarized in Table III. If the criterion of a 100-fold margin of safety on a chronic basis is adhered to, then nordihydroguaiaretic acid and hydroquinone would be considered as deleterious substances for addition to food.

V. SUMMARY

Of the antioxidants listed in the discussion above, evidence of safety for use appears to have been established for propyl gallate, thiodipropionic acid and its dilauryl and distearyl esters, and gum guaiac. Nordihydroguaiaretic acid is not included because no experiments have been conducted in nonrodent species. Hydroquinone must be classed as a harmful and deleterious substance, and as such, has no place as an antioxidant in foods. Catechol has been proposed for antioxidant use (Olcott, 1934), but pharmacological evidence clearly classifies this chemical as a poison.

TABLE III

Pathology in Chronically Poisoned Rats (2-Year Feeding)

Substance	Diet concentration Producing injury	Predominant injury	Source
Nordihydroguaiaretic acid	0.5%	Massive cecal hemorrhages with single and multiple cysts in the mesentery in the angle of junction between small and large intestine	Bieter (1949)
Nordihydroguaiaretic acid	0.5%	Inflammatory cecal lesions and slight cystic enlargement of lymph nodes near the cecum	F & D A† (1950)
Propyl gallate	1.17%	Tubular damage and presence of albuminous casts probably as the result of inanition	Orten *et al.* (1948)
Propyl gallate	5.0%	Patchy hyperplasia in the proventriculus	F & D A† (1950)
Hydroquinone	2.0%	Suggestion of increase in incidence of chronic gastrointestinal ulceration and renal tumors over spontaneous incidence	F & D A† (1950)
* *l*-ascorbyl palmitate * *d*-isoascorbyl palmitate * *d*-isoascorbic acid	0.25% 0.25% 1.0%	No lesions present attributable to the compounds fed	F & D A† (1950)
* Thiodipropionic * Dilauryl thiodipropionate * Distearyl thiodipropionate	3.0% 3.0% 3.0%	No pathological changes	Hazleton (1949)
Catechol	0.25%	Beginning hepatic cell hyperplasia	F & D A† (1950)
* Phenol	1.0%	No pathological changes	F & D A† (1950)
* Gum guaiac	0.5%	No damage attributable to the compound	Bieter (1949)

* Highest concentration fed.
† Food and Drug Administration, unpublished data.

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Studies on Encephalomalacia in the Chick[1,2]

3. THE INFLUENCE OF FEEDING OR INJECTING VARIOUS TOCOPHEROLS AND OTHER ANTIOXIDANTS ON THE INCIDENCE OF ENCEPHALOMALACIA

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DAM *et al.* (1948, 1951) have shown that various antioxidants used in lieu of vitamin E in the diet will afford partial protection to the chick against exudative diathesis and encephalomalacia. Among the substances tested, they found that methylene blue (0.126% of the diet), thionine (0.089%), thiodiphenyl-amine (0.0675%), antabuse (0.025%), nordihydroguaiaretic acid (0.5%), and ascorbic acid (0.5%) afforded considerable, but not complete, protection against exudative diathesis. In the case of encephalomalacia, only methylene blue gave complete protection. Recent work of Singsen *et al.* (1955) has shown that the antioxidant N,N′-diphenyl-p-phenylenediamine (DPPD) was very effective in preventing encephalomalacia. Since DPPD appeared to offer excellent promise in the prevention of this disorder, it seemed advisable to test other antioxidants as to their ability to prevent encephalomalacia when added to the chick's diet at reasonable levels.

Singsen *et al.* (1955) have shown that the primary protective action of DPPD is in the body after the feed is consumed. Since DPPD is a relatively insoluble, poorly absorbed compound, the site of primary action would appear to be the intestinal tract. Injection of DPPD was therefore tried in order to determine its effectiveness when administered by this alternate route. The testing of the various forms of tocopherols was also undertaken for comparison.

EXPERIMENTAL

The general experimental procedure, including the composition of the basal diet, the method of producing vitamin E-deficient chicks, and the methods of tocopherol analysis, has previously been reported by Singsen *et al.* (1954, 1955). The various antioxidants were tested by mixing them directly into the basal diet, which contained vitamin A and D oil as a stress factor. These diets were then fed to lots of from 20 to 24 vitamin E-deficient chicks over a four-week experimental period. In the case of butylated hydroxy anisole (BHA), tests were also run in which the material was incorporated into the oil before mixing into the feed. Since

[1] Supported in part by grants from the encephalomalacia committee of the American Feed Manufacturers Association; The Yantic Grain and Products Company, Norwich, Connecticut; the B. F. Goodrich Chemical Company; and the National Institutes of Health.

[2] Presented at the 43rd Annual Meeting of the Poultry Science Association at West Lafayette, Indiana, July 26–29, 1954.

mortality from encephalomalacia varied from experiment to experiment, a basal ration containing 2% vitamin A and D oil was always fed as a negative control and one containing 2% vitamin A and D oil and a level of DPPD as a positive control. The various antioxidants were tested at the 0.0125% and/or the 0.025% level, with the exception of one experiment in which a level of 0.01% was used.

Any chick showing definite symptoms of encephalomalacia was sacrificed and the brain removed for histological examination. Some chicks, however, either (1) died suddenly without exhibiting typical symptoms, or (2) died during the night when no attendant was on duty. Because of rapid tissue degeneration following death, brain samples were not saved from chicks judged to have been dead more than one hour. In evaluating the results, therefore, one must consider both total mortality and the incidence of encephalomalacia. In the tables the incidence of encephalomalacia is expressed as a fraction, the numerator of which is the number of positive cases of encephalomalacia, and the denominator the number of brains examined.

The DPPD suspension used in one experiment (Table 3) was prepared by the B. F. Goodrich Chemical Company and was a 2.44% suspension of commercial DPPD composed of 240 g. polyethyleneglycol 400 monolaurate, 160 g. polyethyleneglycol 400 dilaurate, 20 g. DPPD, and 400 g. water. In all other experiments, a 1.5% suspension of pure DPPD in 9% Tween-80 was used. This was prepared in our laboratory by adding an acetone solution of DPPD dropwise to a stirred, warmed 9% Tween-80 solution in water. A stream of nitrogen was passed over the surface during the addition and subsequent warming to aid in preventing the oxidation of DPPD. Warming and stirring was continued until all acetone was evaporated from the solution. Since these suspensions gradually settled out on standing it was necessary to shake them well just before use.

N,N′-dipotassium disuccinyl diphenyl-p-phenylenediamine was prepared in our laboratory by reacting diphenyl-p-phenylenediamine with succinic anhydride in the presence of pyridine. The compound was completely soluble in water and a 3.1% solution was prepared for use in the injection experiments.

The water-soluble d-alpha-tocopherol used in these experiments was a d-alpha-tocopherol polyethylene glycol 1000 succinate dissolved in water to give a solution containing 2.5% by weight d-alpha-tocopherol. This solution was prepared by the Distillation Products Industries, Rochester, N. Y. Alpha-tocopheryl acetate cannot be used for injection, because it is not absorbed from the injection site (Blaxter *et al.*, 1953).

The emulsions of d-alpha, -gamma, and -delta tocopherols were prepared as described above for DPPD and contained 2.5% tocopherol in 9% Tween-80 in water.

The vitamin E supplement used in these experiments was a commercial product consisting of d-alpha-tocopheryl acetate adsorbed on defatted soybean meal and standardized to contain 20,000 I.U. of vitamin E per pound.[3]

RESULTS AND DISCUSSION

The antioxidants chosen for study were all commercially available materials, most of which have been tested by Thompson (1950) for their ability to prevent the destruction of carotene in stored alfalfa. Four "hindered phenol" types, two phenylene-

[3] "Myvamix" manufactured by Distillation Products Industries, Rochester, N. Y.

TABLE 1.—*List of antioxidants*

Code No.	Chemical name	Commercial designation
1	2,2-methylene-bis (4-methyl-6-tertiary butyl phenol)	Lederle #2246
2	2,2-ethylene-bis (4-methyl-6-tertiary butyl phenol)	Lederle #425
3	2,6-di-tertiary butyl-4-methyl phenol	BHT, Ionol, dbpc
4	Mixture of 2 and 3 tertiary butyl-4-methoxy phenol	BHA
5	N,N′-di-sec-butyl-p-phenylanediamine	UOP-5
6	N,N′-diphenyl-p-phenylenediamine	DPPD
7	2,5-di-tertiary-butyl hydroquinone	—
8	2,5-di-tertiary-amyl hydroquinone	—
9	Polymer of 1,2-dihydro-2,2,4-trimethylquinoline	—
10	1,2-dihydro-2,2,4-trimethyl-6-phenylquinoline	—
11	1,2-dihydro-2,2,4-trimethyl-6-ethoxyquinoline	Santoquin
12	Thiodipropionic acid	—

diamines, two hydroquinones, three dihydroquinolines, and one sulfur compound were chosen for testing. The chemical names of the antioxidants as well as their commercial names are listed in Table 1. A code number is assigned to each compound and this number is used in subsequent tables to identify the compound. The results of the experiments in which these antioxidants were fed are summarized in Table 2. An interesting fact to be noted is that the commonly used fat antioxidant BHA is apparently of no value in preventing encephalomalacia either when mixed in the oil before the oil is added to the feed or when mixed directly in the feed. At the same time, BHT, which is very closely related in structure to BHA, is apparently as effective as DPPD. Other antioxidants which are apparently equivalent to DPPD in their effectiveness are the dibutyl and diamyl hydroquinones, di-sec-butyl-p-phenylenediamine, and 1,2-dihydro-2,2,4-trimethyl-6-ethoxyquinoline.

TABLE 2.—*The effect of various antioxidants on encephalomalacia*[1]

Our code	Antioxidant, 0.0125%		Antioxidant, 0.025%	
	% mortality	EM.	% mortality	EM.
		Phenolic Type		
1	40	7/8	24	2/2
2	11	1/2	20	1/3
3	5	0/1	5	0/2
4	45	7/9	40	6/6
4 (in oil)			40	3/3
4	38 (.01%)	6/7		
		Amine Type		
5	0	0/0	5	1/1
6	10	1/2	5	0/1
6	4 (.01%)	0/0	14	0/1
		Hydroquinone Type		
7	5	1/1	5	0/0
8	5	1/1	5	0/0
		Dihydroquinoline Type		
9			20	2/3
10			25	3/4
11	5	1/1		
11	0 (.01%)	0/0		
		Sulfur Compound		
12			71	9/11

[1] For a total of 154 chicks in seven different control lots fed the basal ration plus 2% A & D oil, the average mortality was 50.6% and the incidence of encephalomalacia was 57/63.

The various antioxidants which were tested undoubtedly vary in their rate of absorption from the intestinal tract, but the comparative insolubility of DPPD and the observation that there is no detectable absorption from the intestinal tract[4] tend to focus attention on the latter organ as the primary site of action. It is conceivable that some reaction could take place in the intestine, in the presence of unsaturated fats, which produces a toxic byproduct which, in turn, may be absorbed from the intestinal tract and thereby produce encephalomalacia. The presence of the proper antioxidant in the intestine might prevent this hypothetical reaction from occurring. If this premise be true,

[4] "Technical Data on the Preservation of Carotene in Dehydrated Alfalfa" from Research Laboratories of National Alfalfa Dehydrating and Milling Company, Lamar, Colorado, page 22.

then the administration of the antioxidant by another route should not be as effective.

Intramuscular injections of DPPD were therefore tried in order to determine the effectiveness of this antioxidant when administered by an alternate route. The DPPD used for injection in the first experiment was the suspension of the commercial material in various glycol esters and water. Water-soluble d-alpha-tocopherol was also administered by injection as a comparison. Table 3 shows the results of the experiment. In lot 3, vitamin E as d-alpha-tocopheryl acetate was fed in the ration at a level of 8 I.U. per pound of ration. In lot 4, twice-weekly injections of the water-soluble vitamin E were made in the breast or leg muscles of the chicks. An amount of vitamin E was injected which was equivalent to the amount they would have received had they eaten a ration containing 8 I.U. of added vitamin E per pound of ration. In calculating this amount of vitamin E, the average weekly feed consumption per chick from the first through the fourth week was determined from previous experiments. The amount of vitamin E consumed per chick per week was then calculated, and this amount was then divided into two weekly injections. This, of course, assumes 100% absorption of the vitamin E from the feed, and while this is probably not true, this assumption was considered suitable for the purposes of this experiment. The tocopherol blood levels (lot 3 *vs.* lot 4) indicate, however, that this assumption was not too seriously in error. In lot 5, 0.0125% DPPD was fed in the ration; and in lot 6, amounts of DPPD were injected twice weekly, which were equivalent to the amounts they would have received had they eaten a ration containing 0.0125% DPPD. Local necrosis was noted around the site of injection of the DPPD, so that probably either the injection medium or the tars in the commercial DPPD were causing irritation.

TABLE 3.—*Comparison of fed and injected vitamin E versus DPPD at the 8 I.U. per pound and the 0.0125% levels, respectively*

Lot No.	Variable	% mortality	Blood tocopherol, mg. %, 4 wks.	EM.
1	Basal	5	0	1/1
2	Basal+2% oil	90	0	9/11
3	As (2)+8 I.U. Vit. E/lb. in feed	0	0/20	0/1
4	As (2)+Vitamin E injected[1]	0	0.19	0/0
5	As (2)+0.0125% DPPD in feed	5	0	0/1
6	As (2)+DPPD injected[2]	0	0	0/0

[1] Equivalent to amounts the chicks would have received, had they consumed a diet containing 8 I.U. vitamin E per pound.

[2] Equivalent to amounts the chicks would have received, had they consumed a diet containing 0.0125% DPPD.

It is rather remarkable, however, that this highly insoluble compound, when injected as a suspension, did give complete protection against the development of encephalomalacia. A fact worth particular emphasis is that this protection was obtained in spite of a lack of measurable vitamin E in the blood of the chicks, DPPD either fed or injected (lots 5 and 6). This fact suggests that the protective action of DPPD is not confined to the preservation of vitamin E, but that it functions as an *in vivo* antioxidant in a manner parallel to vitamin E and is not limited to an intestinal site of action as postulated above. Therefore, it is apparently unnecessary for DPPD to be in the feed, although there is always the possibility that the injected material could be excreted into the intestine by way of the bile.

Since the amount of DPPD injected was much larger than that of vitamin E, another experiment was designed in which DPPD and vitamin E were compared on a molar basis. In this experiment a sample of pure DPPD was used instead of the commercial form, and the dispersing agent was Tween-80 in water. Also tested was the water-soluble N,N′-dipotassium

disuccinyl DPPD (DPPD-S) prepared in our laboratory. Four I.U. of added vitamin E per pound of ration was used as the "base line" instead of 8 I.U. as in the previous experiment. This amount was chosen because 4 I.U. in the feed is suboptimal, and it was felt that relative differences could be more easily assessed under these conditions. Amounts of vitamin E which were injected were calculated, as before, based on a feed containing 4 I.U. of added vitamin E per pound of ration. Equimolar and five-times-molar amounts of DPPD and equimolar amounts of DPPD-S were also injected. There was no harmful effect from the injection of any of these materials.

The disuccinyl derivative of DPPD was prepared with the idea that solubilizing the DPPD molecule would enable better distribution throughout the tissues with the hope that either the succinyl groups would be hydrolyzed off, freeing the DPPD molecule to perform its function, or that the molecule would still function intact. Although it was realized that an amide linkage with no free hydrogen had only a very slight chance for enzymatic splitting, it was felt that it was worth a trial, since it has been shown that tetrasubstituted phenylenediamines, so-called Wurster dyes, undergo reversible oxidation and reduction to give stable semiquinone radicals (Gilman, 1943). The formation and properties of the semiquinone of DPPD itself have been demonstrated by Linschitz *et al.* (1954). Boyer *et al.* (1949) have shown that tocopherol also forms a moderately stable semi-quinone radical which is reversible to tocopherol. Since there are other examples of semi-quinone formation in physiological processes, it would tend to indicate that semi-quinone formation is undoubtedly of great significance.

The results of this experiment (Table 4) indicate that on an equimolar basis DPPD is apparently not as effective as vitamin E. However, DPPD is probably equivalent, or nearly equivalent, on a weight basis, since there were no cases of encephalomalacia in lot 6 on five-times-molar DPPD, which amount is only three times as much on a weight basis. The comparison, however, is probably not strictly valid, because the comparison is between a completely water-soluble vitamin E and a suspension of DPPD. It may be that the suspension of DPPD is inefficiently absorbed from the injection site and that a principal part of the effect may be derived from the DPPD which is actually in solution in the 9% Tween-80. Analysis of the clear supernatant from a centrifuged DPPD suspension showed it to contain 1.1 mg. DPPD per milliliter of solution. (For method of DPPD analysis, see Bunnell (1954).) More work is under way in this study of injected vitamin E versus injected DPPD.

TABLE 4.—*Comparison on a molar basis of injected vitamin E versus injected DPPD*

Lot No.	Variable	% mortality	EM.
1	Basal+2% oil	80	10/16
2	As (1)+4 I.U. Vitamin E/lb. in feed	5	1/1
3	As (1)+Vitamin E injected[1]	0	0/0
4	As (1)+DPPD-S injected[2]	50	7/7
5	As (1)+DPPD injected[2]	30	3/4
6	As (5), except 5 times as much DPPD	0	0/0

[1] Equivalent to amounts the chicks would have received had they consumed a ration containing 4 I.U. vitamin E/lb.
[2] Molar equivalent to amount in Lot 3.

In the case of DPPD-S, there is some reduction in mortality, but it is not as effective as DPPD. This suggests that either the succinyl groups have inhibited electron exchange, or free hydrogens are necessary for full activity.

In the rat resorption test for vitamin E potency, alpha-tocopherol has the highest activity, with the activity of the other tocopherols decreasing in the order beta, gamma, delta (Embree, 1947). Although delta-tocopherol has only about 1/100th

the biological vitamin E potency of alpha-tocopherol, when compared as antioxidants, delta takes first rank (Stern *et al.*, 1947). Therefore, since antioxidants prevent encephalomalacia, it appeared reasonable to suspect that, for the purpose of preventing this disorder, delta-tocopherol might prove more effective than alpha. Only limited amounts of pure gamma- and delta-tocopherols were available, and since free tocopherols are rather easily destroyed when added to a mixed feed, a preliminary experiment was run using different alpha-tocopherols in the feed.

Table 5 shows the results of a comparison at the 4 I.U. per pound level of d-alpha-tocopheryl acetate, dl-alpha-tocopheryl acetate, and d-alpha-tocopherol. It is readily apparent that there is appreciable destruction of added free tocopherol as evidenced by the higher incidence of encephalomalacia and lower blood tocopherol level for the free tocopherol group. It will also be noted that the d form appears to be better than the dl form; this would be in accord with the biological results with the rat.

Due to this serious loss of tocopherol when fed in the free form, it appeared preferable to test gamma- and delta-tocopherols by injecting them in a 9% Tween-80 emulsion. Table 6 shows the results when equal amounts of these emulsions of pure alpha-, gamma-, and delta-tocopherols were injected twice weekly into the chicks. Amounts injected were

TABLE 5.—*Effect of feeding various alpha tocopherols at the 4 I.U./lb. level*

Lot No.	Variable	% mortality	Blood tocopherol, mg. %, 4 wks.	EM.
1	Basal+2% oil	59	0	6/8
2	As (1)+d-tocopheryl acetate	12	0.42	0/1
3	As (1)+dl-tocopheryl acetate	13	0.27	2/2
4	As (1)+d-tocopherol	44	0.08	3/5

TABLE 6.—*Effect of injecting various tocopherols*[1]

Lot No.	Variable	% mortality	EM.
1	Basal	8	0/0
2	Basal+2% oil	50	7/8
3	As (2)+d-alpha-tocopherol	0	0/0
4	As (2)+d-gamma-tocopherol	8	1/2
5	As (2)+d-delta-tocopherol	46	5/6

[1] Equivalent to amounts the chicks would have received, had they consumed a ration containing 4 I.U. of vitamin E per pound.

calculated, as previously, based on a ration containing 4 I.U. of added vitamin E per pound of ration, assuming equal potency of all three forms. It can be seen that delta-tocopherol gave no protection while alpha-tocopherol gave complete protection. Gamma-tocopherol appeared almost equivalent to alpha-tocopherol.

The mode of action of antioxidants is still obscure, and attempts at explanation are purely in the realm of theory. This work indicates that they are functioning as *in vivo* antioxidants or as electron-transferring agents in some cellular oxidation-reduction step in a manner parallel to vitamin E. Even though it is probably true that the bulk of the DPPD fed in a ration is not absorbed, it would appear that sufficient amounts are absorbed to perform the necessary *in vivo* function. The fact that injections of small amounts of DPPD prevent encephalomalacia would tend to substantiate this. DPPD does protect vitamin E in the semipurified ration (Singsen *et al.*, 1955) used and probably also protects vitamin E in the gut, so that this vitamin E protective action probably adds to its over-all effect when fed in the ration.

The fact that not all antioxidants protect the chick against encephalomalacia militates against a theory that a general antioxidant effect is involved. Since there is little structural similarity between effective antioxidants, including tocoph-

erols, one cannot correlate any specific molecular structure with activity. Therefore, one is left with the possibility that there is a specific range of Eo values, or oxidation-reduction potentials, in which an antioxidant must lie to be effective in preventing encephalomalacia. Dam *et al.* (1951) have also suggested this possibility. A study of the Eo values of the different antioxidants which have been tested might prove illuminating.

It seems, therefore, that in pursuit of the basic cause of encephalomalacia, one is faced with the necessity of delving more deeply into basic cell chemistry. Unsaturated fats are undoubtedly the primary stress factor in producing this disorder, so that the solution probably rests in a greater knowledge of the metabolic pathways of unsaturated fatty acids in normal and in vitamin E-deficient cells. The presence of the proper antioxidant may be necessary to maintain the proper cell potential or to insure a normal metabolic pattern in the cell.

CONCLUSIONS

1. A group of antioxidants were tested for their ability to prevent encephalomalacia in the chick, and several were found to be as effective as diphenyl-p-phenylenediamine (DPPD). Effective antioxidants were: 2,6-ditertiary butyl-4-methoxy phenol (BHT); dibutyl and diamyl hydroquinones; di-sec-butyl-p-phenylenediamine; and 1,2-dihydro-2,2,4-trimethyl-6-ethoxyquinoline (Santoquin).

2. Intramuscular injection of DPPD will prevent encephalomalacia, and, while not equivalent to vitamin E on a molar basis, it was nearly equivalent on a weight basis.

3. By the injection route, delta-tocopherol is ineffective in preventing encephalomalacia, while gamma-tocopherol is nearly equivalent to alpha-tocopherol.

4. It is tentatively concluded that DPPD is functioning as an *in vivo* antioxidant or electron-transferring agent in some cellular reaction which results in the prevention of encephalomalacia. This explanation probably also applies to the other effective antioxidants.

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Studies on Encephalomalacia in the Chick[1,2]

4. THE INFLUENCE OF OIL IN FISH MEAL, OILS FROM VARIOUS SPECIES OF FISH, AND ANIMAL FATS ON THE INCIDENCE OF ENCEPHALOMALACIA

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COD liver oil fed at the 5% level has been demonstrated by Dam (1943) to bring about the symptoms of exudative diathesis and encephalomalacia in the chick when added to a ration known to be deficient in vitamin E and free of fats. Encephalomalacia has been consistently produced in our laboratory by the addition of as little as 2% fortified vitamin A and D feeding oil to a ration deficient in vitamin E and fed to chicks hatched with a low vitamin E reserve (Singsen *et al.*, 1954; Singsen *et al.*, 1955). Dam (1944) has also tested three different fractions of hog liver fatty acids and found that the effectiveness of these acid fractions in producing exudative diathesis and encephalomalacia in chicks reared on a vitamin E deficient diet is related to the iodine values of the acids. Of the fractions studied, the most unsaturated fraction caused the most rapid onset of severe encephalomalacia.

Although encephalomalacia has been demonstrated to be caused by feeding cod liver oil or a fortified vitamin A and D feeding oil, no test has been conducted to compare the encephalomalacia-producing properties of oils from different species of

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[2] Presented at the 43rd Annual Meeting of the Poultry Science Association at West Lafayette, Indiana, July 26–29, 1954.

Effect of Phenolic Antioxidants of Alimentary Fats on the Animal Organism and Their Hygienic Evaluation

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Fats, which represent a highly valuable product of nutrition, are subject to rapid spoilage, which, basically, is the result of oxidizing processes. Oxidation, at low temperatures, is somewhat inhibited, but it is not discontinued; for this reason, even under refrigerating conditions, fats may be preserved no longer than half a year; this cannot satisfy the needs of the food industry.

In fats, during oxidation, there is a disintegration, in the first place, in the fat-soluble vitamins and in the polyunsaturated fatty acids that are required for the normal life activity of the organism. For this reason, the problem concerned with preserving (keeping) fats fresh over a longer period of time also represents a hygienic problem.

In recent years, a proposal has been made to employ chemical methods for protecting fats from spoilage; these consist in introducing into fats substances that have received the designation of antioxidants (antioxidants) ((the latter term in parentheses is an Anglicized version of the word immediately preceding, which bears the same definition -- Translator))

Oxidizing processes in fats proceed in accordance with the chain reaction type. The mechanism involved in the action of antioxidants, apparently, consists in their capacity to break (cut off) the chain reaction; as a result of this, the oxidation process shows a marked inhibition.

The most effective are acknowledged to be phenolic antioxidants, and among them are: butyloxyanisol, butyloxytoluol and gallic acid esters. For an inhibiting action small amounts of the substances are sufficient (0.01 - 0.02%); this makes it possible to increase the storage time of fats by 4 - 10 times. In connection with the proposal to utilize butyloxyanisol, butyloxytoluol and propyl gallate for the stabilization of rendered fats and vegetable oils, the problem arose in the Soviet Union with respect to harmlessness and suitability of such

fats for human nutrition.

There are no domestic studies (papers) on the study of the toxic properties of butyloxyanisol, butyloxytoluol and propylgallate.

The U.S.A., England, Holland, Japan, India and Rumania have been preoccupied with the study of the toxicity of phenolic antioxidants.

Familiarity with these studies (papers) has shown that the LD 50 of butyloxyanisol, butyloxytoluol and of the gallates, in the case of their enteral administration, is a dose above 1000 milligrams/kilogram of the animal's weight; this attests to the comparatively weak toxicity of these compounds. In feeding large amounts (close to 1%, on conversion to the weight of the feed) of these phenolic antioxidants, over a period of 1 - 3 months, the authors have noted an impairmen in the growth and development of the animals. Feeding of propylgallate brought about a diminution in the hemoglobin and morphological changes in the tubular apparatus of the kidneys. The introduction of octyl- and dodecylgallates into the feed of nursing female rats brought about an impairment in lactation and, as a result of this, a deteriorated development of the younger generation.

The majority of researchers, who have conducted experiments on a prolonged feeding of butyloxyanisol, butyloxytoluol and gallates, have come to the conclusion that even that amount of antioxidants, which is 100 - 200 times greater than the one proposed for the introduction of antioxidants into fats, is harmless. However, the conclusions of various authors with regard to the toxicity or harmlessness of the compounds do not always coincide.

Lehman and his co-workers did not observe any harmful effect of propylgallate on rats when feeding it in a dose of 1% in the feed; whereas Orten, Kaiper and Smith consider this concentration to be toxic.

Roy confirms that with a concentration of ethylgallate in fat in an amount of 1%, the animals do not react, whereas Sporn and Peretianu, in the case of a 7.5% content of ethylgallate in the diet's fat, have disclosed a disturbance in the growth of the experimental (test) animals, and a reduction in the utilizati of nitrogen in the nutritional diet. The authors have disclosed dystrophic changes in the thalamus and in the liver.

Consequently, existing literature on the study of the toxic properties of butyloxyanisol, butyloxytoluol and propylgallate does not yield the fully compar ative characteristics of these compounds.

In connection with the inconsistency of the literature data, the present pape should have given an answer to the possibility of utilizing butyloxyanisol, buty oxytoluol and propylgallate for the stabilization of alimentary fats. This was resolved by the following methods.

1. An investigation was made of the organoleptic properties of the products prepared with fat containing antioxidants.

2. Biological experiments were conducted on animals that included determining the degree of toxicity of the preparations in acute experiments, a study of thei toxicity in subacute experiments while feeding large doses, and an investigation of the possible harmful effect on the organism of animals of alimentary fat containing butyloxyanisol, butyloxytoluol and propylgallate in the case of prolonge feeding.

Organoleptic investigations were carried out with the purpose of disclosing t possibility of changes in the external appearance, color, odor and taste of the product, brought about by the addition of the preparation. In the event of disclosing any signs of product spoilage, the problem could be raised as to the possibility of utilizing these compounds in alimentary fats. An undisclosed sampling (tasting) of four samples of fried potatoes, prepared with rendered lar with the addition of 0.02% butyloxyanisol, butyloxytoluol and propylgallate, and without any antioxidants, did not reveal any differences in the organoleptic properties of the potatoes which had been fried with a control sample of fat, ar also, with fat having an addition of antioxidants.

The acute toxicity on the part of the antioxidants, was established for white mice and rats.

The antioxidants were administered to the animals in a solution of sunflower oil directly into the esophagus. 8 rats and 10 mice were inoculated with each dose. Altogether, in the acute experiment, there were used 176 rats and 140 mi

Having carried out an inoculation of the animals with various doses of the

preparations under study, we established the toxicity of these substances from the minimal toxic dose to the absolutely fatal one.

To the extent that it was most convenient to make use of LD 50 for the characteristic of the substance's degree of toxicity, we made a determination of it.

As a rule, the mean lethal dose is determined by means of calculations.

In our investigations, we made a determination of the LD 50 of the preparatior under study by means of the three most widespread methods: by the methods of Berens, Rid-Mench, and by the method of equalizing along a straight line, in ord to clarify whether there would be large disparities between the magnitudes calcu ated by the various methods, inasmuch as many toxicologists and immunologists mak use of methods of integration (those of Berens andRid-Mench), whereas statistica specialists, in particular, Boyarskii, criticize these methods because of the fact that, in integration, there is impaired the randomness (chance) in the sele tion of animals and a systematic error is introduced.

Our results indicate that the lethal doses, for the same compound, calculated by the various methods, do not differ to a marked extent from one another, and i is possible to agree with Prof. Merkov that, for practical purposes, this differ ence has hardly any significance. To the extent that the method of equalizing along a straight line represents the most strictly valid one from the theoretica point of view, and what is most important, does not appear to be more labor-consuming than the methods based on integration, we believe that in experimental studies, the method of equalizing (smoothing out) should be given preference.

The results of acute experiments have shown that the antioxidants under study cannot be reckoned among highly toxic substances. The LD 50 of all three preparations, for rats and mice, were above 1000 milligrams/kilogram of animal weigh

Subacute experiments were set up for the purpose of clarifying on which syste and organs of the animals the antioxidants may have an effect in the case of their daily admission into the organism in large amounts. In the event of disclosing any deviations in the activity of any organs, in these experiments, we wanted to fix particular attention on such tests in chronic experiments in the definitive resolution of the problem concerned with the possibility of introduci

antioxidants into alimentary fats.

Four groups of rats were used in the subacute experiments. Three groups were experimental (test) ones; to them, daily, there were administered, in the feed, antioxidants in an amount of 1/5 of LD 50, i. e., 500 - 600 milligrams/kilogram of the animal's weight. The fourth group was a control one and received pure fa

Fat with antioxidants was fed to animals over a period of 2.5 months; during this period of time, the animals received from 2 to 7 grams of the compounds und investigation.

In the course of the experiment, daily observation was applied to the animals a determination was made of the weight, and a morphological investigation of the blood was carried out; a study was made of the activity of the blood enzymes (of the catalase, peroxidase, and of the cholinesterase); a determination was made c the prothrombin time and a study was made of the urine. At the end of the exper ments the animals were destroyed by decapitation, were dissected, and their internal organs were subjected to a histological investigation. The liver, in addition to a histological investigation, was subjected to a chemical study. In it was determined the content of dry substances, the amount of glycogen, fat and lipoid phosphorus.

The indices of the weight and of the biochemical investigations of the blood and of the liver in the animals were statistically treated; a calculation was made of the degree of reliability in the difference of the indices that had been determined in the case of the experimental (test) and control animals. Weighing of the rats indicated that the introduction, into the diet, of fat with butyloxy toluol brought about, by the 22nd day of the experiment, a lag in the weight of the animals in this group and it was maintained to the end of the experiment. By the 31st day of the experiment, the rats of all three experimental (test) groups (butyloxyanisol, butyloxytoluol and propylgallate) were lagging in weight in comparison with the control group (the difference has a statistical reliability

In the animals of all three experimental (test) groups there was disclosed a reduction in the activity of the blood enzymes. In animals that had received

butyloxyanisol, there was a reduction in the activity of the catalase and of the peroxidase. In rats that had been fed butyloxytoluol, there was a decrease in the activity of all three enzymes under study: of the catalse, peroxidase and of the cholinesterase. The propylgallate showed an inhibiting action on the peroxidase and of the cholinesterase. The propylgallate showed an inhibiting action on the peroxidase and on the cholinesterase.

A reduction in the activity of the oxidation-reduction enzymes in animals that had been fed antioxidants is interesting in view of the fact that these compounds interrupt (discontinue) the oxidation reaction in nutritive (alimentary) products and, apparently, when given in large amounts, manifest a similar action on the flow of the oxidizing processes in the live organism.

In biochemical investigations of the liver, one's attention is drawn to the results of investigations in the butyloxytoluol group. The animals of this group had, in comparison with the control group, a greater liver weight, a greater content of fat in it, and a decrease in the amount of lipoid phosphorus (the reliability has been confirmed statistically). In the animals of this group, apparently, under the influence of administering butyloxytoluol to the organism, there was brought on a disturbance in the synthesis of phospholipids (phosphatides), and, as a result of this, an accumulation of fat in the liver.

There was a somewhat diminished amount of lipoid phosphorus in the liver of rats that had received butyloxyanisol, even though without any retention of fat in this organ.

The histological investigations carried out on the internal organs did not indicate, in the tissues, any changes that could be associated with the administration, into the organism of the animals, of phenolic antioxidants. Only in one rat, that had received propylgallate, was there disclosed an edema of the stroma in a testicle, with the discontinuance of spermatogenesis.

The remaining indices under study, such as the morphological composition of the blood, the prothrombin time, and a study of the urine, did not indicate any changes.

The greatest attention, in the study, was devoted to a study of the possible

toxic action of fats containing antioxidants in chronic experiments. They were carried out on two types of animals: rats and dogs, which remained with the experiment for one year.

As in subacute experiments, the animals were subdivided into four groups. Three of the groups were fed fat with one of the antioxidants, and the fourth group served as a control.

To the extent that with respect to the majority of poisons, a human being is more sensitive than experimental animals, we permitted some aggravation in the chronic experiments (by 10 - 20 times, i. e., an increase in the dose in comparison with the one that may enter the organism of a human being, maximally, with a daily diet).

A feeding that is not fully adequate makes the animals usually more sensitive to the administration (introduction) of harmful agents. For this reason, for a still greater aggravation of the experiment, milk and meat were excluded from the feed diet of rats twice in the course of the year-long experiment (the 7th and 11th months) for 14 days.

Considering the fact that the harmful effect of the substance may show not on the first generation, but on the subsequent ones, we obtained two generations from the principal experimental group, which, prior to attaining a month's growth were separated from the mother and were placed in the experiment. The rats of the second and third generations were included in the experiment for 6 months.

Experiments connected with reproduction are interesting in that, in such physiological states as pregnancy, the sensitivity to poisons increases. By observing females during the period of pregnancy and lactation, we were able to track down the action of antioxidants on the organism, where increased requirements were imposed on it.

In this manner, the possible toxic action of phenolic antioxidants in chronic experiments was studied on 104 rats of three generations and on 8 dogs. Over the extent of the entire experiment, both the rats, as well as the dogs, outwardly appeared to be healthy, active (lively), reacted actively to the environment, and had a good appetite.

The weight of the experimental (test) animals did not differ from the weight of the animals in the control group. Even during that period when the organism was artifically weakened by the elimination of meat and milk from the feed, the change in weight in the experimental (test) animals did not differ from the one in the control group.

We approached the study of the blood enzymes with particular interest; they, before other tests, reacted to the administration, into the organism of the animals, of large amounts of antioxidants, in subacute experiments. These investigations did not reveal any noticeable deviations in the animals that had received butyloxyanisol and butyloxytoluol. In rats to whom propylgallate had been fed, sometimes in an entire group, and sometimes in individual animals, it was possible to detect an increase in the peroxidase activity, in the serum choinesterase in rats of the first generation, in that of the peroxidase -- in animals of the second generation, and in the catalase -- in the third generation

These investigations showed that the feeding of comparatively small doses of propylgallate increases the activity of the blood enzymes; this appears to be undesirable and should be considered a harmful action of the preparation on the organism of the experimental (test) animals.

With the purpose of disclosing any possible impairments in the function of the liver, relative to the synthesis of albumin (protein), an investigation was made of the overall content of albumin in the blood serum, and also, a Takata flocculation test and a Vel'tman (Weltman) coagulation test were set up. No changes were detected by these tests in the amount of serum albumin, in the albumin blood fractions, nor in the electrolytic blood threshold of the experimental (test) animals. Investigations of the remaining nitrogen in the blood of the dogs likewise indicated no pathology in the deaminating and ureagenetic functions of the liver nor any impairments in the excretory capacity of the kidneys.

A study of the indices, which characterize the functional state of the liver such as its glycogen content, and that of fat and lipoid phosphorus, did not disclose any impairments (disturbances) in the activity of this organ in animals that had received butyloxyanisol and butyloxytoluol. Somewhat different results

were obtained in the propylgallate group. In studying the liver of the animals in this group, in two rats there was noted a reduction of the liver (a weight of less than 5 grams), in three there was a reduction in the glycogen content, and another three -- of the lipoid phosphorus. Changes in the liver in the form of diminution in its weight, disturbances in glycogen-formation, or in the synthesi of phospholipids (phosphatides) were noted in 5 animals out of 12 that were participating in the experiment. These observations indicate that the introduction of fat into the diet of rats, along with propylgallate, had a harmful effect on the liver function of part of the experimental (test) animals. Similar changes in dogs had not been disclosed.

The feeding of fat with antioxidants did not indicate any effect on the repro duction of the progeny. Offspring (progeny) were produced both by the experimental(test) as well as by the control animals. Observation of such physiologic indices as the amount of young ones in a litter, their weight on birth, recordin the day of appearance of the incisors, the hairy (furry) coat, and opening of th eyes, disclosed no distinctions in the development of the young generation in a single one of the experimental (test) groups.

A six-month feeding of fat with antioxidants to two generations likewise did not have any influence on their overall state, weight and indices under study, with the exception of changes in the enzymatic activity of the blood; the latter (changes) have already been discussed.

Pathological-anatomical and histological investigations carried out on the internal organs did not reveal any regular (natural) changes that could be considered a result of the administration of antioxidants into the organism of the animals, with the exception of the changes that were disclosed in the sexual system of the males. Atrophy in the testes was disclosed in one animal of the "butyloxyanisol, butyloxytoluol and control" groups. Among rats that had receiv propylgallate, atrophy of the testes was noted in three males. And even though the changes disclosed could be associated with the random (chance) selection of animals with atrophic processes in the tests in the propylgallate group, these data once again put us on guard relative to the harmful action of propylgallate.

Thus, the experimental investigations carried out on a prolonged feeding of fat with phenolic antioxidants to three generation of rats and dogs did not indicate any harmful action onthe part of butyloxyanisol and butyloxytoluol on the live organism, whereas propylgallate was not neutral (indifferent) withrespect t the experimental (test) animals.

Our data, relative to propylgallate, have something in common with the recent terminated investigations of Profs. Budagyan and Smirnova, who, having studied the assimilability of nutritive (alimentary) fat, containing the same antioxidants as in our experiments, have indicated that the assimilability of fat is som what reduced upon including, in the diet, fat with propylgallate, whereas the introduction, into the feed, of butyloxyanisol and butyooxytoluol, did not manifest any effect.

The study thus carried out makes it possible to draw the conclusion that the introduction of butyloxyanisol and butyloxytoluol in the alimentary fats, with the purpose of inhibiting, in them, the oxidizing processes, finds no objections in hygienic attitudes, whereas the use of propylgallate cannot be resolved.

It should be also noted that the introduction of antioxidants into fats may b resolved at those enterprises in which a laboratory or appropriate institute may carry out a daily control over the amount of preparation introduced.

The introduction of antioxidants into fats should not in any wise reflect on carrying out and searching for new measures for preventing the oxidation of fats in the course of production and storage. To the same degree as previously, attempts should be made not to permit any prolonged storage of raw material, to pressurize (seal hermetically) the fat-rendering processes to the greatest exten possible, and carry out the storage of fat at a low temperature. Only these measures, in conjunction with antioxidants, will make it possible to increase considerably the storage time of fats without reducing their full physiological value.

Translated by Carl Demrick Associates, Inc./LB/db

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REVUE ROUMAINE DE BIOCHIMIE

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EFFECT OF THE ANTIOXIDIZER BUTHYLHYDROXYANISOL (BHA) ON THE RESPIRATION AND OXIDATIVE PHOSPHORYLATION

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Experiments were performed in 80 young, white rats, divided into 10 groups in order to study the influence of the alimentary antioxidizer buthylhydroxyanisol (BHA), administered for 8 weeks, on the endogenous respiration, on the O_2 uptake in presence of succinate as substrate, and on the oxidative phosphorylation of the hepatic mitochondria. It has been established that BHA in doses of 0.1% and 1% determines a significant increase in the O_2 uptake, in presence of succinate as substrate. The 1% BHA dose has influenced also the phosphorylation efficiency of the hepatic mitochondria, the P/O ratio being significantly increased as compared to the control group. The P/O ratio is increasing proportionally to the antioxidizer dose administered.

In previous researches [14] we found that BHA administered to white rats for eight weeks in a 1% concentration, induces a significant change of the succinic dehydrogenase activity. The question arose if the BHA is acting only on this enzyme or is also modifying the oxygen uptake in the presence of the succinate as substrate. Taking into account the fact that 5–6% of the BHA is eliminated as such, unmetabolized [3, 6], and that the antioxidizer is rediscovered in the fat perirenal tissue [9], the hypothesis has been advanced that the substance could act at the cell level, influencing in the first stage the oxido-reducing processes [5, 11]. Bearing in mind at the same time that BHA is eliminated particularly as glucuronoconjugate (46% in rabbits and 61–82% in rats) and sulphoconjugate (9% respectively 11–16%) [3, 6] and that these detoxifying processes imply the presence of the ATP as energy source, it has been considered that in the case ATP synthesis would be affected by the antioxidizer, disorders could occur in the detoxifying processes, and that would contribute to the appearance of some toxic effects [5]. Placer Z. and coll. [11], finding that the antioxidizers are

blocking the dehydrogenase activity of the hepatic homogenate, *in vitro*, have advanced the hypothesis that blocking of the energy transfer from the respiratory chain to the ATP, could occur inducing an inhibition of the respiration in mitochondria and of the ATP synthesis.

As these hypotheses are not supported by any data, we have thought to tackle this side of the problem by studying the effect of BHA administration on the hepatic homogenate respiration in the presence of succinate as substrate, and on the oxidative phosphorylation in white rats. Choosing the oxidative phosphorylation ratio (P/O) as indicator, we took into account the essential importance of this transforming process of the energy mobilized in various exergonic reactions in energy stored in the ATP molecule, as well as that the high energy bonds synthesis represents the biochemical process having the highest efficiency.

The study of the ATP synthesis in cells became feasible due to the possibility of investigating the mitochondria structure.

MATERIAL AND METHODS

Experiments were performed in 80 young, white rats (Wistar), of similar age and weight (approx. 40 g) divided into 10 groups. The animals were kept for 4 weeks, respectively 8 weeks, in individual cages, receiving a diet with the following content per 100 g : 18 casein, 12 lard, 60 corn starch, 4 Osborne-Mendel salts mixture, 2 agar-agar, 4 dry yeast. Weekly 270 I.U. A vitamin, 1 mg E vitamin and 100 I.U. D_2 vitamin, were administered *per os*. The antioxidizer has been included in the diet fat of the experimental groups, in 0.1, 1 and 8.3% concentration. At the end of each experimental stage the animals were killed by decapitation. The O_2 uptake has been determined on 10% hepatic homogenate in 0.25 M mannitol, by the manometric conventional Warburg technique. The basic reaction mixture contained in 2 ml volume : 15 mM kalium phosphate buffer (pH 7.4) ; 6.7 mM magnesium sulphate ; 25 mM kalium chloride ; 0.1 mM adenosintriphosphoric acid ; 0.02 mM cytochrome C. 0.5 ml hepatic homogenate 10% have been included in the main compartment, 0.1 ml substrate 10 mM was added in the side-arm. After the thermoequilibration at 30°C, the side-arm substrate was tipped in and the O_2 uptake was measured every 10 minutes. The results obtained, expressed in μl, have been considered with respect to 50 mg wet liver. Endogenous respiration (using H_2O instead of the substrate) and the O_2 uptake, using succinate as substrate, have been also measured. Rat liver, extracted immediately after the killing and introduced in the homogenizing mixture, cooled beforehand to 0°C, has been used in order to obtain mitochondria. The liver has been cut in slices and washed several times with the homogenizing mixture (mannitol 0.3 M). Homogenizing has been carried out in a glass homogenizer with plexiglass piston, Börnig and Richter pattern [4] The homogenate (about 15%) has been centrifugated for 10 minutes (600 g) and, after the supernatant decantation, the residue was taken again, with 2 mixture volumes, and mildly homogenized. After another 10′ centrifugation at 600 g, both supernatant suspensions were mixed up and centrifugated again for 7′ (600 g). Mitochondria have been deposited by a 20′ centrifugation (4000—5000 g) and washed once (or twice) in the homogenizing mixture. The washed particles have been suspended in mannitol 0.23 M — saccharose 0,07 M — TRIS 0.03 M (pH 7.4).

Hexokinase has been prepared from fresh yeast, by the Sols and coll. method [12]. The enzyme is maintained several months at —20°, though a gradual decrease of activity occurs.

Oxidative phosphorylation has been estimated as the difference between the content in inorganic phosphate (Pi) of the initially inactivated vessels (at zero stage) and the content of the inactivated vessels at the mentioned stages, by the method indicated by Gurban [8].

The inorganic phosphate has been measured by the Fiske — Subbarow method [7]. For estimating the Pi concentration a scale on pure solutions comprising 3 to 27 moles Pi in solution has been traced. Assay of mitochondrial suspension proteins was carried out by the Aldrige method [2]. Albumin, fraction V N.B.C., has been employed as standard.

The uptake of O_2 (ΔO_2) has been expressed in μ atoms and that of Pi (Δ Pi) in μ moles. The phosphorylation ratio (P/O) has been expressed by the ratio between the esterified Pi as μ moles and the uptaken O_2 μ atoms per the quantity of protein employed in each experiment.

RESULTS

Endogenous O_2 uptake. The administering of BHA for four weeks has induced a decrease in the endogenous respiration, but although at 0.1% BHA dose the differences reach 36% in comparison with the control group, they are not statistically significant (Table 1). The same effect occurred also after 8 weeks administration, the differences being still smaller (19%).

Table 1

Influence of BHA administration on the respiration

Groups	Endogenous respiration in μl			O_2 uptake in presence of succinate in μl		
	Mean	SE*	p**	Mean	SE	p
	Experiment I: 4 weeks administering					
I Control	42	6.1		106	7.5	
II BHA 0.1%	27	5.6	>0.05	113	10.3	>0.05
III BHA 1%	37	4.9	>0.05	114	7.6	>0.05
	Experiment II: 8 weeks administering					
IV Control	49	9.3		126	9.6	
V BHA 0.1%	40	6.8	>0.05	102	3.8	<0.02
VI BHA 1%	47	8.0	>0.05	98	3.9	<0.003

* SE = average standard error

** p = Pearson's test

The O_2 uptake in the presence of succinate as substrate. The administering for 4 weeks of BHA in 0.1% and 1% concentration has not influenced significantly the O_2 uptake in the presence of succinate (Table 1). After 8 weeks a significant decrease of the total O_2 uptake was recorded, proportional to the BHA dose administered. We recorded at 0.1% dose a decrease of 20% ($p < 0.02$) and at 1% dose a decrease of 23% ($p < 0.003$) (Table 1).

Administration of BHA for 8 weeks has determined an inhibition of the O_2 uptake (Fig. 1). The differences between the groups having

received 0.1 and 1% BHA were minimum. After 10 minutes the respiration rate, in the groups having received BHA, presents the maximum difference as compared to the respiration rate of the control group; subsequently there is a tendency to diminish the differences which continue however to be statistically significant (Fig. 1).

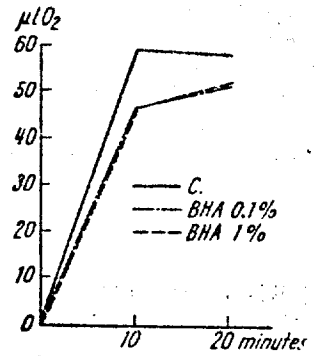

Fig. 1. — Respiratory rate of rat liver homogenate in presence of succinate.

Oxidative phosphorylation. The study of the obtained results (Table 2) shows that BHA administration for 8 weeks in 1% concentration has influenced the phosphorylation efficiency of the hepatic mitochondria, the P/O ratio being significantly increased as compared to the control group ($p < 0.05$).

Studying the two components of the ratio, i.e. the O_2 up-take and the Pi esterification, we find minimum and insignificant differences between the experimental groups and the respective control group. The analysis of the phosphorylation rate shows also the absence of significant changes in the group having received BHA as compared to the control group. These results permit the conclusion that at similar rates of phosphorylation the output has been greater in the group having received 1% BHA.

Given the fact that the differences between the control group and the one having received 1% BHA, though statistically significant, have been relatively small (10%), we have repeated the experiment making

Table 2

Influence of BHA administration for 8 weeks on oxidative phosphorylation

Group		ΔO* Mean	SE	p	ΔPi** Mean	SE	p	P/O*** Mean	SE	p
VII	Control	8.49	0.21		15.82	0.54		1.86	0.05	
VIII	BHA 1%	8.20	0.36	>0.05	16.64	0.58	>0.05	2.06	0.09	<0.05
IX	Control	9.6	0.36		18.5	0.86		1.92	0.04	
X	BHA 8.3 %	8.5	0.51	>0.05	19.3	0.67	>0.05	2.31	0.12	<0.001

* Expressed in μ atoms
** " " μ moles
*** Ratio between μ moles Pi and μ atoms O.

the experimental conditions more severe by increasing the dose of BHA to 8.3%. The results obtained show that BHA administration has determined an increase of the P/O ratio without influencing the O_2 uptake and the Pi esterification. The increase of the P/O ratio has been proportional to the BHA dose administered, the differences being of 20% as compared to the control group.

DISCUSSION

The results obtained confirm the hypothesis that BHA influences the oxidoreducing processes at the cell level. This is illustrated by the fact that BHA, in presence of succinate, determines a significant inhibition of the O_2 uptake. Correlating this fact with the results of our previous researches [14], in which the antioxidizer administration has determined significant modifications of the succinic dehydrogenase activity, we can conclude that the action of BHA is not exerted on a single complex, from the four ones of the respiratory chain, but is acting on the whole chain.

The fact that a significant decrease in the O_2 uptake is recorded also with the 0.1% dose in presence of succinate, proves the increased sensitivity of this metabolic chain to the action of BHA, and also that this dose, being ten times greater than the usual one, is not indifferent to the organism. This conclusion is sustained also by the results of our previous researches [13], demonstrating that a significant decrease of the leucocytes and opsonines phagocytosis capacity has been recorded with the 0.1% BHA dose. We must stress the fact that the effects determined by BHA manifest themselves relatively slowly. So, while after 4 weeks period no indicator presented significant modifications, a significant decrease of the O_2 uptake was recorded after 8 weeks. It could be presumed that the BHA action appears only after a certain storing of the antioxidizer in the tissues, the antioxidizer being accumulated in the perirenal fat tissue [9]. As the dynamics of the antioxidizer storing does not increase, it is not possible to appreciate if it is a so-called cumulative phenomenon or a level accumulation. Elucidation of this aspect of the problem is very important in order to appreciate the antioxidizer toxicity. After 4 weeks, a 0.1% concentration of BHA determined an inhibition of the endogenous respiration (36%), but which was not statistically significant. This inhibition could be explained as an influence of the antioxidizer on the endogenous substrate formed as a result of the lipides hydrolysis [10]. In order to elucidate this point further researches are required. Our results show that the 0.1% concentration of BHA is not indifferent to the organism. The problem arises if the effects obtained are specific to BHA only or are common for any antioxidating substance. To elucidate this point further researches are necessary, which have to cover also other antioxidizers of varied antioxidating power and toxicity. Some interesting indications concerning the toxicological significance of the modifications produced by BHA result from the analysis of the antioxidizer influence on the oxidative phosphorylation. The results show that BHA determines a stimulation of the phosphorylation efficiency, therefore of the ATP synthesis. This stimulation of the ATP synthesis could be correlated with the increased need for ATP in the course of the detoxifying processes of BHA in the organism. Analysing the modifications determined by the 1% BHA dose in the oxidoreducing processes and of oxidative phosphorylation at mitochondria level, it can be concluded that they belong to the limits

of the reactivity possibilities of the organism and do not reflect proper toxic effects. Nevertheless the possibility is not excluded that the modifications recorded determine secondary toxicological effects, as, for instance, the decrease of the immunological reactivity, recorded in our previous researches [13].

From the practical point of view it is necessary to revise the maximum concentrations admissible for BHA, considering the dose of 0.1–0.5 mg/kg body not as a dose to be accepted without reserve as the FAO/WHO [1] commission proposes, but as a dose imposing some rezerve. Taking into account that the 0.1% dose in fat represents 0.01% in the diet and that this dose is equivalent to one of 5 mg/kg body, it results that the 0.05 mg/kg dose is not indifferent to the organism. Therefore a dose to be accepted without any reserve cannot be established in the case of BHA, but, as also for BHT*, only a limit to be accepted with some reserves.

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COMPTE RENDU

GHEORGHE HARALAMBIE, *Biochimie şi sport* (La Biochimie et le sport), Ed. Uniunii de Cultură Fizică şi Sport, Bucarest, 1966, 229 p., 49 fig., 41 tabl.

Dans les conditions actuelles de développement du mouvement sportif de notre pays, l'apparition d'un matériel de base aidant à la compréhension des processus biochimiques qui ont lieu pendant l'effort était tout à fait impérieuse. L'apparition de ce travail, le premier dans notre pays et l'un des très rares parmi ceux du monde entier, tente à remplir le vide dans ce domaine. La tâche que l'auteur s'est assumée a été particulièrement difficile de plusieurs points de vue. Ainsi, il a été nécessaire que les aspects biochimiques soient traités d'une manière accessible, vu que le travail est adressé à une gamme variée de lecteurs. De même, vu la complexité des aspects traités, il a été nécessaire le choix attentif du matériel scientifique. Pour ces raisons, un tel livre ne pouvait être écrit que par un auteur ayant une vaste expérience didactique et professionnelle, ce qui heureusement s'est produit.

Le livre contient neuf chapitres où sont décrits quelques processus biochimiques, tout en étant soulignés les processus affectés pendant l'effort. Le premier chapitre a un caractère introductif, et contient la description des aspects biochimiques généraux, une brève histoire du développement de la biochimie, les méthodes d'investigation, la manière d'interprétation de certaines données biochimiques et les erreurs possibles ; dans les IIe et IIIe chapitres on présente les sources d'énergie et la manière dont celle-ci est produite pendant l'activité physique.

Les chapitres IVe et V^e contiennent la description du mode de production d'énergie dans le métabolisme des principales substances génératrices d'énergie, les trois principaux métabolismes et l'équilibre ionique.

Dans les chapitres suivants, VIe — IXe, on décrit des problèmes spéciaux, en relation directe avec le sport, notamment les particularités biochimiques en fonction du type d'effort effectué, les manifestations biochimiques à caractère négatif pendant l'effort, le rétablissement et la super-compensation des facteurs biochimiques, ainsi que des caractéristiques biochimiques de « l'état d'entraînement ».

Tout le matériel est traité d'une façon très claire permettant ainsi aux lecteurs la compréhension des processus biochimiques, difficilement abordables pour des non-spécialistes. Il faut également apprécier la riche bibliographie, où sont mentionnés de nombreux travaux des biochimistes roumains.

Le livre « La biochimie et le sport » est un succès et en même temps le premier essai de combler un domaine où s'impose une plus ample activité éditoriale de spécialité.

Dinu Tănăsescu

Effect of the Antioxidants of Alimentary Fats -- Propylgallate and Butyloxyanisol -- on the Animal Organism.

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UDK (Universal Decimal Classification):

613.268 / 613.288:664.3.033.5

Fats, and the products that they contain, are subject to rapid spoilage principally because of the oxidation of fats even upon storing them at a temperature below 0 deg. Highly unsaturated fatty acids and fat- soluble vitamins, with a high physiological activity, are oxidized in the first place.

Papers have made their appearance that attest to the toxic action of a number of oxidation products, such as peroxides, aldehides, ketones and polymers. This explains the interest that is manifested in processes that take place in fats and in the methods of protecting them from spoilage.

The chemical process is one of the most effective methods of protecting fats from oxidation. For this purpose, recently, there have been proposed a great number of compounds and their combination; they have been given the designation of antioxidants ((Anglicized version of the Russian word -- Translator)) or antioxidants ((Russian version of the same word -- Translator)). However, many of them do not satisfy the strict requirements of physicians and of healths supervision agencies. An illustration of the foregoing may be found in the figures (statistics) set forth by Kraybill and Dugan (1): in the U.S.A. alone, in 10 years (from 1944 to 1954), there have been granted 600 patents for proposals of substances and their combination in the capacity of antioxidants of alimentary fats. Antioxidants and their various combinations are employed for addition to nutritive products in more than 20 countries. In many of them, such as in the U.S.A., the South African Republic, Finland, Sweden, Yugoslavia and Canada, there is utilized a considerable amount of various antioxidants (up to 25 designations). In the Soviet Union, there has been permitted the use of two antiooxi-

dants in food products: butyloxyanisol and butyloxytoluol.

Many antioxidants may intensify their effect in conjunction with other substances. This synergism is also manifested in mixtures of various antioxidants. In studying various mixtures of antioxidants, Gearhart and Stuckey (2) came to the conclusion that the best is represented by a combination of butyloxyanisol (BOA) and propylgallate (PG). N. Emanuel', D. Knorre, Yu. Lyaskovskaya and V.Piul'skaya (3) have shown that the most synergistic effect is manifested by PG in a concentration of 0.005% , and BOA -- in 0.01%. Kraybill and Dugan (1) discovered that if, to 0.01% BOA, there is added 0.003% PG, and 0.002% citric acid, then the stability of the fat under study will increase by two times in comparison with fat containing only BOA, and by 13 times in comparison with control fat without any antioxidants.

The possibliility of adding a small amount of antioxidants (as a result of their synergism) is very tempting. I. Smorodintsev believes that it is more feasible (practical) to add the least possible amount of phenolic anti-oxidants, b to intensify their action through the introduction of one or several synergists.

Graham and his co-authors (1) studied a mixture of TENOKS-2, consisting of 20% BOA, 6% PG, 4% citric acid and 70% propylene glycol (as a solvent). Other groups of rats were fed doses of TENOKS-2 that were increased, in comparison with possible penetration into the human organism, by 6 and 300 times. It was disclosed that a combination of BOA and PG did not manifest any toxic action on the animals; the latter had been observed for a period of a year. A mixture of TENOKS-2 has received permission to be used in the U.S.A. for the purpose of stabilizing fish oil, and also, fat in pork and in the meat of turkeys(turkey hens).

The data published in the foreign literature with regard to the toxicity of a mixture of BOA and PG antioxidants are insufficient, in view of the fact that, in conformity with the materials set forth, the action of the mixture was observed only for some general biological indices.

In connection with the foregoing, and also, for the reason that in the U.S.S.R., the toxicity of the mixture was not studied, for the complete toxicological characteristics of the mixture of antioxidants, we carried out acute, subacute and chronic experiments.

The purpose of the acute experiments was to determine the LD_{50} of the mixture.

Inoculation was carried out with a solution of antioxidants (the ratio of BOA to PG was 2 : 1). The inculation mixture consisted of 20% BOA, 10% PG and 70% sunflower oil in the capacity of a solvent. The inoculation was accomplished on 7 groups of white male rats (with 10 animals in each group; the average weight of the animals was 100 - 120 grams) by means of administering the mixture of antioxidants directly into the stomach through a metallic probe. The LD_{50} was calculated by the method of Miller and Teitner[1]. The results are set forth in Fig. 1. ((Graph on pg. 77 of text)) ((underneath this graph)): Fig. 1. Determination of the LD_{50} by the method of probit (probability unit)-analysis. On the axis of ordinates, there is a linear expression of the the probits (probability units), and on the axis of abscissas, above -- the doses being tested (in grams/kilogram), and below -- the result obtained (number of animals that has succumbed). CIRCLE -- graphic expression for the LD_{50} of the mixture of anitoxidants. ((text continued)):

The LD_{50} obtained for the mixture of antioxidants is equal to 2633 plus or minus 140 milligrams/kilogram; it proved to be between the LD_{50} for BOA (2950 milligrams/kilogram) and that for PG (2500 milligrams/kilogram). These data have shown that a mixture of BOA and PG, in acute experiments, does not manifest any synergistic action and that a mixture of BOA and PG may be figured among the weakly toxic substances.

Subacute experiments were carried out on young white male rats and on females having a weight of 100 - 120 grams. In the experiment were 40 animals (20 males and 2 0 females.) The purpose of the subacute experiments was a study and selection of the most sensitive tests for carrying out chronic experiments.

The feeding was carried out over a period of $2\frac{1}{2}$ months. A mixture of antioxidants was given in rendered lard with a No.1 ground feed briquet. The amount of antioxidants was aggravated by 500 times (500 - fold) in comparison with the amount that could find its way into the human organism: BOA - 100 milligrams/kilogram; PG -- 50 milligrams/kilogram.

In the course of the experiment, observations were carried out daily o the interrelationship between the consumption of the feed and the external appearance of the animals in the experimental and control groups; the weight was determined every 10 days.

Proceeding from the fact that antioxidants, in the live organism, man fest, possibly, an effect on the oxidation processes, we carried out an investigation of the exit-oxygen of the blood, and also, of the blood catalase and kcholinesterase activity.

In breaking off the oxidizing processes in its or in inhibiting them, the antioxidants could exert an influence on the fat metabolism in the organism of the experimental (test) animals; for this reason, we made a study of the amount of ketone bodies in the blood. In addition to this, the diastase of the urine and the sugar of the blood were subjected to investigation.

The animals remained in the experiment from 60 to 70 days. Investigations were carried out on the 20th, 40th and 60th day of the inoculation. In the treatment of the material, those results in which P is equal to or less than 0.05 were taken as being statistically reliable.

Over the extent of the entire experiment, no changes were observed in the animals with respect to the behavior, external appearance and the consumption of their feed. Experimental (test) and control animals added weight equally well. On the 20th day of the inoculation, there was some reduction in the activity of the cholinesterase (P is less than 0.02). Subsequently, evidently, an adaptation took place on the part of the organism to the introduction of antioxi-

dants, and the difference between the animals in the experimental and control groups leveled off (Table 1) . Such an effect was disclosed only in females. In the males, a difference between the control and experiment was not noted.

Investigations of the catalase activity indicated that the antioxidants, in the beginning, inhibit the action of this enzyme (on the 20th day), and after that, its activity increases (on the 40th day). These shifts were also noted in the females (Fig. 2).

((Table on pg. 78 of text))

Table 1

Blood Cholinesterase Activity

Animals	Days of investigation	Control			Experiment						
		n	M	m_M	n_o	M_o	m_M	M_o-M	m_M-M	t	P
Males	Prior to the experiment										
	20										
	40										
	60										
Females	Prior to the experiment										
	20										
	40										
	60										

((See Table 1 on pg. 78 of text for all figures pertaining thereto))

((Two illustrations on pg. 78 of text)) ((to the left of the upper illustration)):
Catalase activity; ((immediately underneath this illustration)):
((from left to right)) Prior to the experiment; 20th day of the experiment; 40th day of the experiment; 60th day of the experiment; ((below that)): Fig.2. Catalase activity expressed in the catalase index (females).
Dark circles -- experiment; light circles - control; continuous line -- average (mean) magnitude. ((to the left of the lower illustration)): Blood sugar (in milligram %); ((immediately underneath this illustration)): ((from left to right)): Prior to the experiment; 20th day of the experiment; 40th day of the experiment; 60th day of the experiment; ((below that)): Fig. 3.

Blood sugar (males). The legend (conventional) designations) is the same as it is in Fig. 2. ((text continued)):

An analogous action dynamics was disclosed (by us) relative to the blood sugar: in the beginning there takes place a diminution in the amount of suga and after that, an increase in it (Fig. 3). These changes were observed only in the males. In the females , there was no difference between the experiment and the control.

On the 40th day of the inoculation, we disclosed a statistically reliable increase in the ketone/bodies in the males (P is less than 0.02), and a statistically reliable reduction in the ketone bodies in the females (P is less than 0.02). These data are set forth in Table 2. Changes in the exit - oxygen of the blood were not noted.

((Table on pg. 79 of text))

Table 2

Ketone Bodies in the Blood

Animals	Days of Investigation	Control n	Control M	Control m_M	Experiment n_o	Experiment M_o	Experiment m_M	$M-M_o$	$m_{M_o}-M$	t	P
Males	Prior to the experiment										
	20										
	40										
	60										
Females	Prior to the experiment										
	20										
	40										
	60										

((See Table 2 on pg. 79 of text for all figures pertaining thereto))

((text continued)): The antioxidants likewise showed no effect on the pancreas: investigations of the diastase activity of the urine of the lipase activity, directly in the pancreas, showed no difference between the animals in the control and experimental groups.

Following the experimental period, all the animals were destroyed by decapitation. Investigations carried out on the liver glycogen did not disclose any differences between the control and the experiment. The ratio of the weight of the liver to the weight of the body showed a tendency to an increase in this ratio in the experimental animals (P is less than 0.1).

Conclusions

1. BOA (Butyloxyanisol and PG (propylgallate), individually, and also a mixture of these antioxudants, do not pertain to strongly toxic substances.

2. In the action of the mixture of BOA and PG antioxidants on the organism, in acute experiments, there is no disclosure of synergism, nor of antagonism.

3. Large concentrations of the mixture of antioxidants (BOA -- 100 milligrams/kilogram and PG -- 50 milligrams/kilogram) are not neutral (indifferent)for the blood organism. They exert an influence on the blood catalase and cholinesterase activity, and on the amount of ketone bodies and blood sugar ; this attests to their negative action on the organism.

4. The white rat females proved to be more sensitive to the administration of a mixture of antioxidants than did the males.

5. In order to permit the application of BOA and PG antioxidants (in mixture) in alimentary fats and in fat-containing products, it is necessary to carry out investigations under conditions of chronic inoculation of the animals; and this is what represents the subject of investigation at the present time.

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Footnote

(a) Quoted from L.M. Bien'kii, Elements in the Quantitative Evaluation of the Pharmacological Effect, L(eningrad), 1963, p. 63.

(Translated by Carl Demrick Associates Inc. LB/fs)

Figure 1

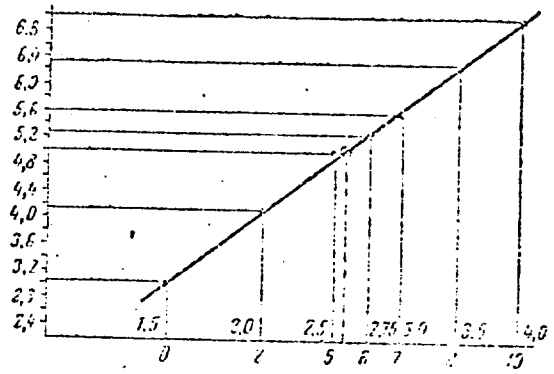


Figure 2

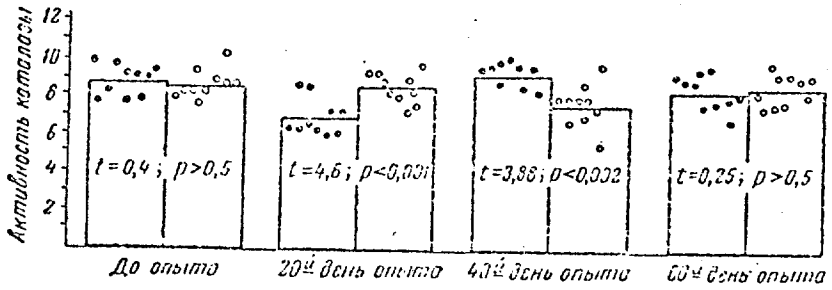
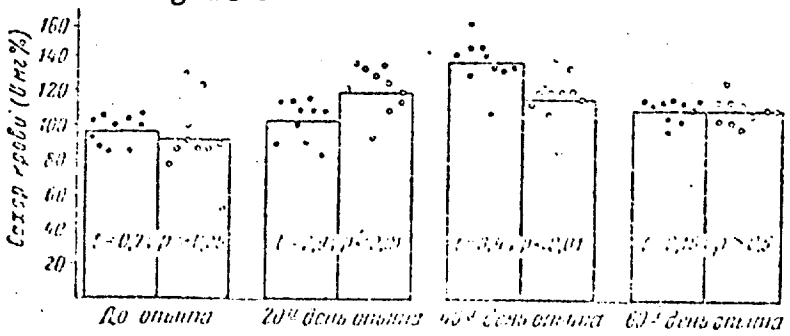


Figure 3



Активность холинэстеразы крови

Животные	Дни исследования	Контроль			Опыт			M_0-M	m_{M_0-M}	t	P
		n	M	m_M	n_0	M_0	m_M				
Самцы	До опыта	10	1,22	0,024	10	1,23	0,02	0,01	0,032	0,31	>0,5
	20	10	1,26	0,02	10	1,26	0,015	0	0,022	0	—
	40	10	1,27	0,02	10	1,27	0,012	0	0,016	0	—
	60	10	1,32	0,02	10	1,34	0,014	0,02	0,026	0,79	>0,25
Самки	До опыта	10	1,30	0,03	10	1,31	0,007	0,01	0,03	0,3	>0,5
	20	10	1,33	0,007	10	1,30	0,003	0,03	0,011	2,5	<0,02
	40	10	1,29	0,01	10	1,29	0,007	0	0,012	0	—
	60	10	1,34	0,03	10	1,35	0,033	0,01	0,043	0,25	>0,5

Table 1

Кетоновые тела крови

Животные	Дни исследования	Контроль			Опыт			$M-M_0$	m_{M_0-M}	t	P
		n	M	m_M	n_0	M_0	m_M				
Самцы	До опыта	10	9,02	1,87	10	10,04	1,7	1,02	2,62	0,4	>0,5
	20	10	8,35	1,77	10	11,56	0,999	3,21	2,02	1,57	>0,1
	40	10	5,94	1,30	10	14,90	3,03	8,96	3,32	2,66	<0,02
	60	10	8,53	1,70	10	10,40	0,92	1,87	1,90	0,9	>0,25
Самки	До опыта	10	7,68	0,50	10	8,09	0,60	0,41	0,81	0,2	>0,5
	20	10	6,63	0,919	10	7,27	0,26	0,64	1,24	0,51	>0,5
	40	10	10,00	1,16	10	6,27	0,90	3,73	1,43	2,6	<0,02
	60	10	7,86	0,81	10	8,60	0,40	0,74	0,91	0,81	>0,25

Table 2

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УДК 613.268+613.288]:664.3.033.5

ВЛИЯНИЕ АНТИОКИСЛИТЕЛЕЙ ПИЩЕВЫХ ЖИРОВ ПРОПИЛГАЛЛАТА И БУТИЛОКСИАНИЗОЛА НА ЖИВОТНЫЙ ОРГАНИЗМ

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Жиры и содержащие их продукты подвергаются быстрой порче главным образом из-за окисления жиров даже при хранении их при температуре ниже 0°. Окисляются в первую очередь высоконепредельные жирные кислоты и жирорастворимые витамины с большой физиологической активностью.

Появляются работы, свидетельствующие о токсическом действии ряда продуктов окисления, таких, как перекиси, альдегиды, кетоны, полимеры. Это и объясняет заинтересованность, которая проявляется к процессам, происходящим в жирах, и способам их защиты от порчи.

Одним из наиболее эффективных способов защиты жиров от окисления является химический. За последнее время для этой цели предложено множество соединений и их сочетаний, получивших названия антиоксидантов или антиокислителей. Однако многие из них не отвечают строгим требованиям врачей и органов санитарного надзора. Иллюстрацией к изложенному выше могут служить цифры, приведенные Крейбилом и Дуганом [1]: только в США за 10 лет (с 1944 по 1954 г.) было выдано 600 патентов на предложения веществ и их сочетаний в качестве антиокислителей пищевых жиров. Антиокислители и различные их комбинации применяются для добавления в пищевые продукты более чем в 20 странах. Во многих из них, таких, как США, ЮАР, Финляндия, Швеция, Югославия, Канада, используется значительное количество различных антиокислителей (до 25 наименований). В Советском Союзе разрешено применение в пищевых продуктах двух антиокислителей: бутилоксианизола и бутилокситолуола.

Многие антиокислители могут усиливать свой эффект в сочетании с другими веществами. Этот синергизм проявляется и в смесях различных антиокислителей. Исследуя различные смеси антиокислителей, Герхарт и Стакей [2] пришли к выводу, что наилучшей является комбинация бутилоксианизола (БОА) и пропилгаллата (ПГ). Н. Эмануэль, Д. Кнорре, Ю. Лясковская и В. Пнульская [3] показали, что наибольший синергетический эффект ПГ проявляет в концентрации 0,005%, а БОА — в 0,01%. Крейбил и Дуган [1] нашли, что если к 0,01% БОА прибавить 0,003% ПГ и 0,002% лимонной кислоты, то стабильность исследуемого жира увеличится вдвое по сравнению с жиром, содержащим один БОА, и в 13 раз по сравнению с контрольным жиром без антиокислителя.

Возможность добавлять меньшее количество антиокислителей (благодаря их синергизму) весьма заманчива. И. Смородинцев считает, что целесообразнее добавлять как можно меньшее количество фенольных антиокислителей, но усиливать их действие введением одного или нескольких синергистов.

Грахам и соавторы [4] изучали смесь ТЕНОКС-2, состоящую из 20% БОА, 6% ПГ, 4% лимонной кислоты и 70% пропиленгликоля (как растворителя). Двум группам крыс скармливали дозы ТЕНОКС-2, увеличенные в сравнении с возможным попаданием в орган[illegible] человека в 6 и 300 раз. Было выявлено, что комбинация БОА и ПГ не [illegible]вала токсического действия на животных, наблюдение за которыми велось в течение года. Смесь ТЕНОКС-2 разрешена к применению в США для стабилизации рыбьего жира, а также жира в свином мясе и мясе индеек.

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Опубликованные в зарубежной литературе данные о токсичности смеси антиокислителей БОА и ПГ недостаточны, так как, согласно представленным материалам, наблюдалось действие смеси лишь на некоторые общебиологические показатели.

В связи с изложенным выше, а также потому, что в СССР токсичность смеси не изучалась, для полной токсикологической характеристики смеси антиокислителей нами проведены острые, подострые и хронические опыты.

Целью острых опытов было определение LD_{50} смеси.

Затравку проводили раствором антиокислителей (соотношение БОА к ПГ — 2 : 1). Затравочная смесь состояла из 20% БОА, 10% ПГ и 70% подсолнечного масла в качестве растворителя. Затравку осуществляли на 7 группах белых крысцамцов (по 10 животных в каждой группе, средний вес животных 100—120 г) путем введения смеси антиокислителей непосредственно в желудок через металлический зонд. LD_{50} была рассчитана по методу Миллера и Тейтнера[1]. Результаты представлены на рис. 1.

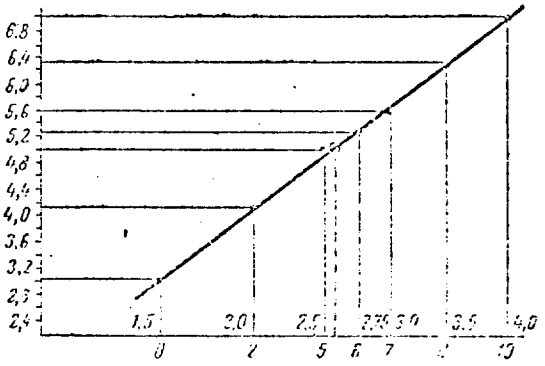

Рис. 1. Определение LD_{50} методом пробит-анализа. На оси ординат — линейные выражения пробитов, на оси абсцисс сверху — вводимые дозы (в г/кг), снизу — полученный результат (число погибших животных).

О — графическое выражение LD_{50} смеси антиокислителей.

Полученная LD_{50} для смеси антиокислителей равна 2633±140 мг/кг; она оказалась между LD_{50} БОА (2950 мг/кг) и ПГ (2500 мг/кг). Эти данные показали, что смесь БОА и ПГ в острых опытах не проявляет синергетического действия и что смесь БОА и ПГ может быть причислена к слаботоксичным веществам.

Подострые опыты были проведены на молодых белых крысах-самцах и самках весом 100—120 г. В опыте было 40 животных (20 самцов и 20 самок). Цель подострых опытов — изучение и выбор наиболее чувствительных тестов для проведения хронических опытов.

Скармливание проводили в течение 2½ месяцев. Смесь антиокислителей давали в сыром топленом жире с молотым кормовым брикетом № 1. Количество антиокислителей затравировали в 500 раз по сравнению с количеством, которое может попасть в организм человека: БОА — 100 мг/кг; ПГ — 50 мг/кг.

В течение опыта ежедневно проводились наблюдения за взаимосвязью между поеданием корма и внешним видом животных опытной и контрольной групп, вес определялся через каждые 10 дней.

Исходя из того, что антиокислители в живом организме, возможно, оказывают влияние на процессы окисления, мы исследовали вакат-кислород крови, а также активность каталазы и холинэстеразы крови.

Прерывая окислительные процессы в жирах или тормозя их, антиокислители могли повлиять на жировой обмен в организме подопытных животных, поэтому мы изучали количество кетоновых тел в крови. Кроме того, исследованию были подвергнуты диастаза мочи и сахар крови.

Животные находились в опыте от 60 до 70 дней. Исследования проводились на 20-й, 40-й, 60-й день затравки. При обработке материала за статистически достоверные результаты принимались те, в которых $P < 0{,}05$.

На протяжении всего опыта у животных не было отмечено изменений в поведении, внешнем виде и поедании корма. Подопытные и контрольные животные одинаково хорошо прибавляли в весе. На 20-й день затравки несколько снижалась активность холинэстеразы ($P < 0{,}02$). В дальнейшем, видимо, происходила адаптация организма к введению

[1] Цит. Л. М. Беленький. Элементы количественной оценки фармакологического эффекта. Л., 1963, стр. 63.

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антиокислителей, и разница между животными опытной и контрольной групп нивелировалась (табл. 1). Такой эффект был выявлен только у самок. У самцов разница между контролем и опытом не была отмечена.

Исследования каталазной активности показали, что антиокислители вначале ингибируют действие этого фермента (на 20-й день), а затем

Таблица 1

Активность холинэстеразы крови

Животные	Дни исследования	Контроль			Опыт			M_0-M	m_{M_0-M}	t	P
		n	M	m_M	n_0	M_0	m_M				
Самцы	До опыта	10	1,22	0,024	10	1,23	0,02	0,01	0,032	0,31	>0,5
	20	10	1,26	0,02	10	1,26	0,015	0	0,022	0	—
	40	10	1,27	0,02	10	1,27	0,012	0	0,016	0	—
	60	10	1,32	0,02	10	1,34	0,014	0,02	0,026	0,79	>0,25
Самки	До опыта	10	1,30	0,03	10	1,31	0,007	0,01	0,03	0,3	>0,5
	20	10	1,33	0,007	10	1,30	0,003	0,03	0,011	2,5	<0,02
	40	10	1,29	0,01	10	1,29	0,007	0	0,012	0	—
	60	10	1,34	0,03	10	1,35	0,033	0,01	0,043	0,25	>0,5

его активность возрастает (на 40-й день). Эти сдвиги также отмечены только у самок (рис. 2).

Аналогичную динамику действия мы обнаружили в отношении сахара крови: вначале происходит уменьшение количества сахара, а затем

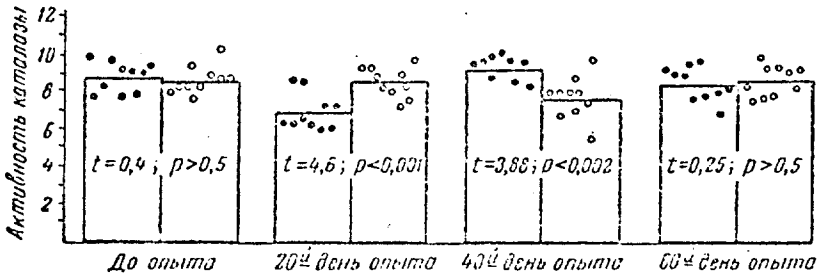

Рис. 2. Активность каталазы, выраженная в показателе каталазы (самки).

Темные кружки — опыт; светлые кружки — контроль; сплошная линия — средняя величина.

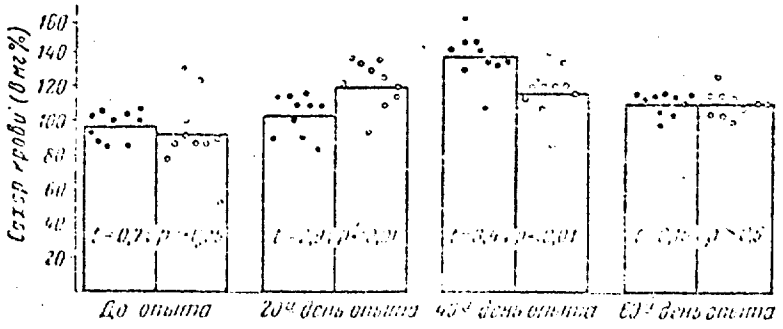

Рис. 3. Сахар крови (самцы). Условные обозначения те же, что на рис. 2.

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увеличение (рис. 3). Эти изменения наблюдались только у самцов. У самок разницы между опытом и контролем не было.

На 40-й день затравки нами было обнаружено статистически достоверное увеличение кетоновых тел у самцов ($P<0,02$) и статистически достоверное уменьшение кетоновых тел у самок ($P<0,02$). Эти данные представлены в табл. 2. Изменений в вакат-кислороде крови не было отмечено.

Таблица 2

Кетоновые тела крови

Животные	Дни исследования	Контроль			Опыт			$M-M_0$	m_{M_0-M}	t	P
		n	M	m_M	n_0	M_0	m_M				
Самцы	До опыта	10	9,02	1,87	10	10,04	1,7	1,02	2,62	0,4	>0,5
	20	10	8,35	1,77	10	11,56	0,999	3,21	2,02	1,57	>0,1
	40	10	5,94	1,30	10	14,90	3,03	8,96	3,32	2,66	<0,02
	60	10	8,53	1,70	10	10,40	0,92	1,87	1,90	0,9	>0,25
Самки	До опыта	10	7,68	0,50	10	8,09	0,60	0,41	0,81	0,2	>0,5
	20	10	6,63	0,919	10	7,27	0,26	0,64	1,24	0,51	>0,5
	40	10	10,00	1,16	10	6,27	0,90	3,73	1,43	2,6	<0,02
	60	10	7,86	0,81	10	8,60	0,40	0,74	0,91	0,81	>0,25

Антиокислители также не оказали влияния на поджелудочную железу: исследования диастазной активности мочи и активности липазы непосредственно в поджелудочной железе не показали разницы между животными контрольной и опытной групп.

После опытного периода всех животных забивали обезглавливанием. Проведенные исследования гликогена печени не выявили различий между контролем и опытом. Отношение веса печени к весу тела показало тенденцию к увеличению этого отношения у опытных животных ($P<0,1$).

Выводы

1. БОА и ПГ в отдельности, а также смесь этих антиокислителей не относятся к сильнотоксичным веществам.

2. В действии смеси антиокислителей БОА и ПГ на организм в острых опытах не проявляется ни синергизм, ни антагонизм.

3. Большие концентрации смеси антиокислителей (БОА — 100 мг/кг и ПГ — 50 мг/кг) не безразличны для организма. Они влияют на активность каталазы и холинэстеразы крови, количество кетоновых тел и сахара крови, что свидетельствует об их отрицательном действии на организм.

4. Самки белых крыс оказались более чувствительными к введению смеси антиокислителей, чем самцы.

5. Для разрешения применения антиокислителей БОА и ПГ (в смеси) в пищевых жирах и жиросодержащих продуктах необходимо проведение исследований в условиях хронической затравки животных, что и является предметом исследования в настоящее время.

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THE EFFECT PRODUCED BY PROPYLGALLATE AND BUTYLOXYANISOL — ALIMENTARY FATS ANTIOXYDANTS — ON THE. ANIMAL ORGANISM

M. A. Daniyalov (Moscow)

Summary

Acute and subacute experiments were instituted to study the toxicity of mixed antioxydants — propylgallate and butyloxyanisol (taken in proportion of 1:2). The LD_{50} of the mixture was found to equal 2633±140 mg/kg. In subacute tests the animals were given a mixture of antioxydants aggravated 300-fold as compared to a possible penetration into the human organism (butyloxyanisol — 100 mg/kg, propylgallate — 50 mg/kg). The mixture of the studied antioxydants produced a negative effect of the blood enzymes—catalase and cholinesterase, as well as upon the ketone bodies and blood sugar.

УДК 614.31:[63+631.42]:615.778.3-099-014.3+614.76:615.778.3-014.3

ПРИМЕНЕНИЕ ТОНКОСЛОЙНОЙ ХРОМАТОГРАФИИ ДЛЯ ОПРЕДЕЛЕНИЯ ГЕПТАХЛОРА, ДДТ И ЛИНДАНА В РАСТИТЕЛЬНОМ МАТЕРИАЛЕ И ПОЧВЕ

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В нашей стране для борьбы с вредителями сельскохозяйственных культур широко применяются различные инсектициды, в том числе и хлорорганические. Известно, что многие хлорорганические инсектициды обладают резко выраженными кумулятивными свойствами, т. е. опасны с точки зрения хронического отравления ими. В связи с этим большую актуальность приобретает определение этих ядохимикатов в пищевых продуктах и почве. Однако установить наличие их в биологических объектах очень трудно из-за отсутствия приемлемых методов очистки экстракта от веществ, мешающих определению инсектицидов.

В недавно появившейся работе Эббота и Томпсона [1] указывается способ очистки пестицидов на пластинке, имеющей форму трапеции, в тонком слое сорбента силикагель — кизельгур. Быстрота, эффективность и простота выполнения побудили нас применить тонкослойную хроматографию для очистки экстракта, содержащего гептахлор, ДДТ и линдан. Сорбентом служил силикагель марки «КСК». Очистку экстракта осуществляли на стеклянной пластинке трапециевидной формы. Преимуществом такой формы пластинки является то, что наряду с очисткой она позволяет и концентрировать инсектициды. Из неокрашенных зон силикагеля инсектицид элюировали н-гексаном.

В элюате представлялась возможность определять содержание инсектицидов общеизвестными методами (хроматографическим, колориметрическим, спектрофотометрическим и др.).

Приготовление пластинок с закрепленным слоем сорбента

Силикагель марки «КСК» размалывают на шаровой мельнице или растирают в ступке. Размолотый силикагель обрабатывают соляной кислотой (1 : 1); через сутки кислоту декантируют и силикагель заливают свежей порцией кислоты. Эту операцию

повторяют до тех пор,
гель отмывают дистилл
тем дважды промывают
ванной водой (до нейтр
ют метанолом при нагр
силикагель подсушивают
верстий 0,04—0,06 мм.
ликагеля с величиной
фракцию, но с величиной
при 120°, затем охлажда
товленный силикагель м
Слой силикагеля н
ристого кальция (20%)
ра осадок промывают с
в течение 48 часов при 1
Для очистки обра
следующих размеров:
5 см. На пластинку на
готовленную из расчета
стинки. Пластинку расп
был толще (до 300 мк).
в термостат на 1 час при
Разделение вещест
рые наносят силикагель
силикагеля и 3 г гипса с
ют в склянке с притерто
вынимают, при этом на
ния хлороформа (через
жат (сорбентом вниз) и
вируют 10 мин. в сушиль

Экстракция инсекти

Навеску исслед
количеством безводн
бу и заливают петр
бы навеска была по
встряхивания на 1
отделяют от расте
сы или почвы. Остат
промывают петроле
ром. Слитый в одну
ракт концентрируют
мом. Концентриров
тяжку (около 3—5 м
зуют для идентифик
химикатов и колич
их определения.
С этой целью н
материал предварит
щают в тонком слое
ля путем нанесения
на трапециевидную
линией, параллельно
края (рис. 1).
Ширина линии
достигать 1,5—2 см,
образцами очистке
держащий инсектиц
пластинку помещают
15 мин. В результате
остаются на старте,

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CHRONIC TOXICITY OF BREAD ADDITIVES TO RATS. PART II

By W. Donald Graham and H. C. Grice

From the Food and Drug Laboratories, Department of National Health and Welfare, Ottawa, Canada

Received October 18, 1954

In an earlier investigation[1] of the chronic toxicity of certain bread additives the ingredients tested (chlorine dioxide, polyoxyethylene (8) monostearate, sodium propionate and a mixture of propyl gallate and butylated hydroxyanisole) were incorporated in bread at 50 times the normal concentration and used for 52 weeks at 75 per cent. of the diet of rats. Under the conditions of the experiment no adverse effects were noted and these results were at variance with some of the experimental evidence obtained by other investigators, particularly in regard to the polyoxyethylene (8) monostearate[2,3,4,5]. Possibly this difference was due to experimental technique. It was of interest, therefore, to retest the bread ingredients previously studied, with the difference that they were not mixed with water, baked into bread, and the bread dried and ground but were added directly to the basal ration.

Diet I contained the ingredients of an essentially normal bread (75 per cent.) added to a basal medium supplying minerals, vitamins, protein, fat, and bulk (25 per cent.). In diet II, flour treated with 50 times the normal amount of chlorine dioxide, lard containing 50 times the usual level of antioxidants, and sodium propionate and polyoxyethylene (8) monostearate each at 50 times the usual concentration were incorporated in the bread ingredient portion of the diet. Diets III, IV, V and VI were similar to diet II, except that one ingredient in each, respectively emulsifier, mould inhibitor, antioxidants, or flour improver, was reduced to the normal level. Diet VII was a commercial cubed ration ground to a consistency similar to that of the other diets. Diets were mixed at intervals of no greater than 2 weeks and were kept in closed cans in the refrigerator. The details of diet composition for convenience are reproduced from the previous publication[1] in Table I.

A group of 210 Wistar strain male albino rats ranging in age from 23 to 36 days and in weight from 35 to 79 g. was divided into 14 sub-groups by arranging them in descending order of weight and randomly assigning the rats in successive lots of 14 to the 14 sub-groups. These sub-groups, each consisting of 15 animals, were assigned to the 7 diets yielding two series, called for convenience replicates A and B, and numbered according to the number of the diet given. The oldest rats had been maintained on diet VII from weaning at 22 days of age until placed on test. The 14 groups of rats were housed in cages with wire screen floors. Each cage held 1 group (15 rats). Cages were moved in a regular rotation on the racks so that all spent approximately equal intervals at the 4 different levels above the floor. Food and water were permitted *ad libitum*. Records of weekly weight, food consumption and mortality

TABLE I

DIET COMPOSITIONS

Part A.—75 per cent. of the diet.

Ingredient	Diet I	II	III	IV	V	VI	VII
Commercial bread flour[1]	70·5	—	—	—	—	70·5	GROUND MASTER
Bread flour 50 × ClO_2[1]	—	70·5	70·5	70·5	70·5	—	
Commercial lard[2]	1·8	—	—	—	1·8	—	
Lard 50 × Antioxidants[2]	—	1·8	1·8	1·8	—	1·8	
Sodium propionate[3]	0·1	5·0	5·0	0·1	5·0	5·0	
Polyoxyethylene (8) monostearate[4]	0·3	15·0	0·3	15·0	15·0	15·0	
Cerelose (glucose)	20·4	0·8	15·5	5·7	0·8	0·8	
Salt (NaCl)	1·8	as I	as I	as I	as I	as I	
Skim milk powder	2·2	"	"	"	"	"	
Malt flour	0·5	"	"	"	"	"	
Yeast food[5]	0·2	"	"	"	"	"	
Wytase[6]	0·9	"	"	"	"	"	
Yeast	1·3	"	"	"	"	"	
	100·0	100·0	100·0	100·0	100·0	100·0	

B.—25 per cent. of the diet.

Ingredient	Diet I	II	III	IV	V	VI	
Casein	60·0	as I	as I	as I	as I	as I	FOX CUBES
Alphacel[7]	9·4	"	"	"	"	"	
Corn oil	12·8	"	"	"	"	"	
Mineral mix[a]	12·0	"	"	"	"	"	
Vitamins and excipient[b]	5·8	"	"	"	"	"	
	100·0	100·0	100·0	100·0	100·0	100·0	

[a] Minerals—the 12·0 g. of mineral mix present in 100 g. of basal diet were made up as follows:—

KCl	1·580	$FePO_4$	0·195
KH_2PO_4	4·100	$KAl(SO_4)_2.12H_2O$	0·001
$Ca_3(PO_4)_2$	1·970	$CuSO_4.5H_2O$	0·080
$MgSO_4$	1·190	NaF	0·076
$MnSO_4.H_2O$	0·027	KI	0·001

[b] Vitamins—the 5·8 g. of vitamins and excipient in 100 g. of basal diet were made up as follows:—

Thiamine hydrochloride	0·0020 g.	Folic acid	0·0008 g.
Riboflavine	0·0040 g.	*p*-Aminobenzoic acid	0·0040 g.
Calcium pantothenate	0·0080 g.	Liver fraction‡	0·8000 g.
Pyridoxine	0·0020 g.	Menadione	0·0004 g.
Nicotinamide	0·0080 g.	E concentrate*	0·0560 g.
i-Inositol	0·0800 g.	A and D concentrate†	0·1600 g.
Choline chloride	0·4000 g.	Corn oil	4·2830 g.

* Vitamin E concentrate, 350 mg. of *d*, α-tocopheryl acetate equivalent per g.
† Navitol, 65000 A, 13000 D per g.
‡ Wilson's Liver fraction L.

NOTES

[1] Chlorine dioxide.—Canadian No. 1 patent flour was treated with 0·3 g. of chlorine dioxide per barrel (196 lb.) to yield commercial bread flour and with 15·0 g. of chlorine dioxide per barrel to yield high ClO_2 flour.

[2] Antioxidants.—Tenox II, a commercial preparation containing 20 per cent. of butylated hydroxyanisole, 6 per cent. of *n*-propyl gallate, 4 per cent. of citric acid and 70 per cent. of propylene glycol was used at the rates of 0·05 per cent. in commercial lard and 2·5 per cent. in high antioxidant lard.

[3] Propionate.—The commercial propionate sold by Du Pont de Nemours and Co., under the trade name Mycoban was used.

[4] Polyoxyethylene monostearate.—The material sold by Atlas Powder Co. as polyoxyethylene (8) monostearate under the trade name Myri 45 was used.

[5] Yeast food.—The commercial material sold under the name RKD by Standard Brands was used.

[6] Wytase.—This material is sold by J. R. Short Canadian Mills, Ltd., and is used commercially to improve whiteness.

[7] Alphacel is a commercial cellulose supplying bulk without nutritive value.

were kept. Where the condition of the carcass warranted it, detailed autopsies were performed on animals that died on test; in other instances advanced autolytic changes precluded accurate decision as to the cause of death.

After 16 weeks on the diets 2 rats selected from each group by random choice were killed and a number of tissues were preserved for histological

study. Blood samples from each of these rats were analysed for hæmoglobin content by a slight modification of the pyridine hæmochromogen method of Rimington[6]. After 32 weeks on the diet only 3 rats survived in group II A and the experiment was terminated. All surviving rats were killed and the liver, left kidney, heart and spleen were weighed. Tissue samples were reserved for histological examination and subsequently sections from cerebellum, cerebrum, myocardium, trachea, œsophagus, thyroid, salivary gland, thymus, peri-bronchiolar lymph gland, lung, stomach, small intestine, liver, spleen, adrenal, pancreas, kidney, bladder and testis were studied.

RESULTS

The mean body weights at intervals during the 32-week feeding trial of the rats in the 14 groups are presented graphically in Figure 1. The mean body weights of the survivors on each diet and the standard errors of the means are shown for replicates A and B and for the replicates combined in Table II.

TABLE II

FINAL WEIGHT OF SURVIVORS AT 32 WEEKS
Weight in g. ± standard error

Diet	Replicates combined		Replicate A		Replicate B	
	Mean weight	Number	Mean weight	Number	Mean weight	Number
I	296 ± 7·4	16	297 ± 12·4	7	295 ± 9·6	9
II	258 ± 7·4	13	257 ± 21·8	3	259 ± 13·2	10
III	268 ± 10·3	15	277 ± 13·5	7	260 ± 15·4	8
IV	281 ± 9·4	21	273 ± 14·2	11	290 ± 10·9	10
V	252 ± 7·0	21	247 ± 10·0	11	258 ± 9·9	10
VI	263 ± 6·5	18	255 ± 10·0	10	274 ± 7·0	8
VII	292 ± 8·1	19	305 ± 16·2	7	283 ± 8·4	12

TABLE III

ANALYSIS OF VARIANCE OF FINAL WEIGHT DATA

Main effect	DF	Mean square	F
Replicates	1	335	1
Diet	5	4,345	4·4*
Replicates X diet	5	792	1
Error	111	984	—

* Significant at P = 0·01.

Growth was depressed, particularly during the first few weeks on diets II, III, V and VI, i.e. on those diets containing the high level of sodium propionate. A detailed examination of the data revealed that the total weight gain subsequent to the first 4 weeks on the diet was approximately the same for each diet and the apparent depression of the final weights on these 4 diets high in propionate was the result of the curtailed growth during the first few weeks. Growth rates based on weight changes between the 5th and 32nd weeks were not significantly influenced by diet. Growth on diet I was very similar to that on diet VII throughout the test.

Statistical analysis of the final weight data for diets I to VI using Snedecor's methods for disproportionate groups[7] revealed a significant

(P = 0·01) influence of diet on final weight (Table III). The effect was mainly that of the high level of sodium propionate, for growth on diet IV was not significantly depressed.

Cumulative feed consumption per rat per day over the 32-week period is shown in Figure 2. As in the previous study[1] the rats ate more of the stock diet (VII) than of any of the test diets. In this experiment, feed consumption figures for all diets were higher than the corresponding

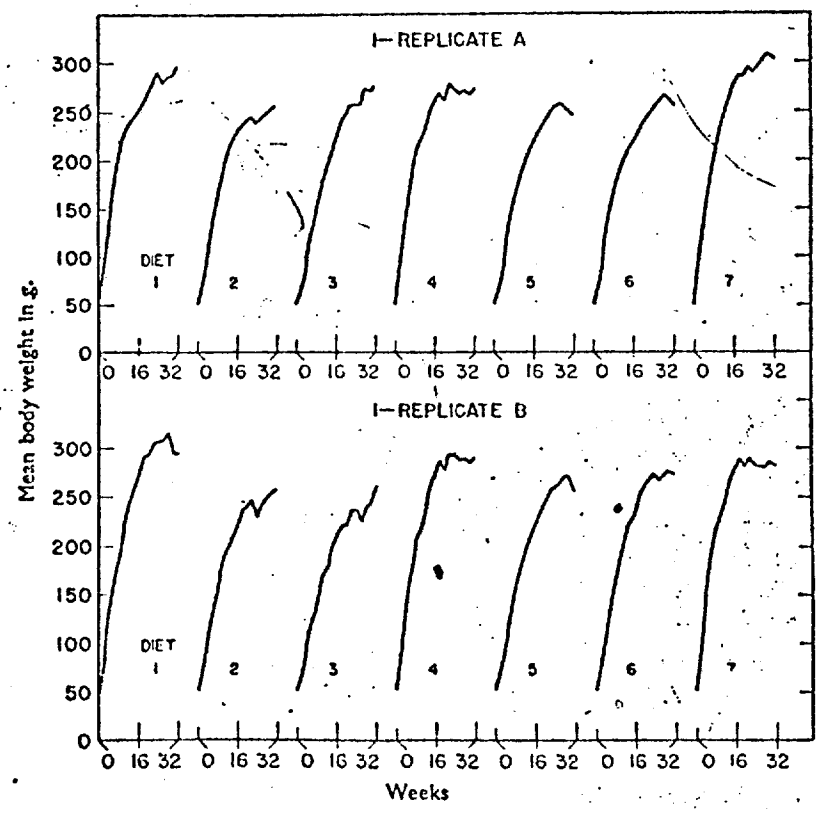

FIG. 1. Mean body weight of the rats in the 14 groups.

figures from the earlier data by an average of some 8·5 per cent. Mean body weights overall were only 3 per cent. higher. These differences are reflected in the generally poorer feed efficiency also depicted graphically in Figure 2. The striking effect of diet which may be observed here is the very poor feed efficiency during the first weeks on diets II, III, V and VI, particularly on diet III.

Curtailed growth, slightly lower feed consumption, and markedly lowered feed efficiency on diets II, III, V and VI during the early weeks indicate that sodium propionate fed at high levels to the young rat had a deleterious effect. That this effect was not solely due to lack of palatability of the diet is obvious from the fact that feed efficiency was much

more seriously affected than was feed consumption. Although the data are not amenable to statistical assessment, they suggest that polyoxyethylene (8) monostearate at high levels in conjunction with high sodium

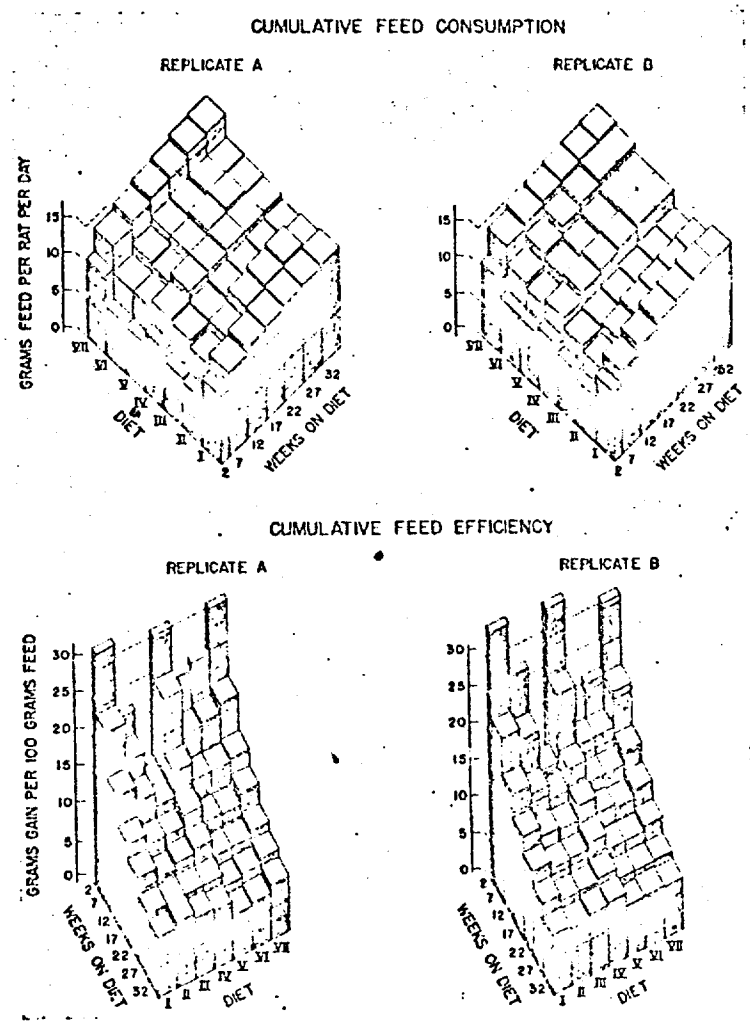

FIG. 2. Cumulative feed consumption per rat per day over the 32-week period.

propionate (diets II, V and VI) increased the efficiency with which the high propionate diet was utilised by young rats (compare diet III). Apparently the rats became acclimatised to the high propionate diet, for growth rate, feed consumption and feed efficiency approached the control levels within a few weeks.

The hæmoglobin levels in the blood of the 28 rats killed at the end of 16 weeks were all within the normal range for rats in this colony.

Sections from the tissues of 151 rats were stained with hæmotoxylin and eosin and examined microscopically. As in the previous investigation[1] the groups to which the individual rats belonged were unknown

TABLE IV

HISTOPATHOLOGICAL FINDINGS

	Replicate													
	A							B						
Diet	I	II	III	IV	V	VI	VII	I	II	III	IV	V	VI	VII
Number of rats examined*	9	5	9	13	13	12	9	11	12	10	12	12	10	14
Normal	4	2	4	2	0	1	1	5	4	1	5	1	1	3
Trachea—inflammation	–	–	–	–	5	2	–	2	–	1	–	4	1	–
Lung—respiratory infection	2	1	–	5	10	7	6	2	1	1	3	8	7	6
Lung—papilloma of bronchus	1	–	–	–	–	–	–	–	–	–	–	–	–	–
Thymus—inflammation	–	–	1	–	–	1	–	–	–	–	–	–	–	–
Stomach—gastritis	–	–	1	–	–	–	–	–	2	–	–	–	–	–
Stomach—erosion	–	–	–	–	–	–	–	–	1	–	–	1	–	–
Adrenal—congestion	–	–	–	1	–	–	–	–	–	1	–	–	–	–
Kidney—tubal necrosis	–	–	–	–	1	–	–	–	–	–	–	–	–	–
Kidney—nephritis	–	1	–	1	1	–	–	–	–	–	–	1	–	–
Kidney—congestion	–	–	–	–	–	–	–	–	–	1	–	1	–	–
Liver—cloudy swelling	–	–	1	2	1	–	–	–	–	–	2	–	–	–
Liver—venous congestion	–	–	–	1	–	–	–	–	1	–	–	–	–	–
Pancreas—fatty degeneration of acinar tissue	–	–	–	–	–	–	–	–	–	–	–	–	1	–
Intestine—enteritis	–	–	1	–	1	–	–	–	1	–	–	–	–	–
Intestine—papilloma	–	–	–	–	–	–	–	–	–	1	–	–	–	–
Testis—reduced spermatogenesis	–	–	–	–	–	–	–	1	–	–	–	–	–	–
Bladder—parasitic cysts	4	3	5	8	5	6	8	5	5	9	6	4	6	8

* 2 rats were killed at 16 weeks, the remainder at 32 weeks.

MORTALITY DATA

Died on test	6	10	6	2	2	3	6	4	3	5	3	3	5	1
Autopsy	3	4	5	2	0	2	3	2	1	4	2	3	4	0
Undiagnosed	3	6	1	0	2	1	3	2	2	1	1	0	1	1
Respiratory infection	1	4	2	–	–	2	1	1	1	4	2	3	3	–
Middle ear disease	2	–	3	1	–	–	–	1	–	–	–	–	2	–
Encephalitis } Probable extension from middle ear infection	2	–	4	–	–	–	–	–	–	–	–	–	–	–
Meningitis	–	–	1	1	–	–	–	1	–	–	–	–	2	–
Pericarditis	–	–	1	–	–	–	1	–	–	–	–	–	1	–
No visible lesion	–	–	–	1	–	–	1	–	–	–	–	–	–	–

to the pathologist at the time the tissues were studied. The histopathological findings are presented in Table IV and have been summarised by the pathologist as follows:

Findings in the cerebrum, cerebellum, myocardium, œsophagus, thyroid, salivary gland, thymus, small intestine, spleen, adrenal, pancreas, stomach and testis were negative or of no significance. Most of the lungs exhibited some changes due to pneumonia, including emphysema, bronchiectasis and atelectasis, although only acute and sub-acute cases have been listed in Table IV. The predominant bronchopneumonia invariably was accompanied by mild chronic inflammatory changes in the trachea. The two instances in which congestion and polymorphonuclear infiltration of the peribronchiolar lymph nodes were observed probably were the result of infection from the lungs. While cloudy swelling of the liver is the first sign of a degenerative process caused by

bacterial or chemical toxins, the few cases noted here were very mild and of doubtful significance. The incidence of kidney abnormality is similar to that reported previously[1], and again these abnormalities were observed in rats on diets II, III, IV and V, those diets high in chlorine dioxide. The incidence of the kidney changes again is too low to warrant any conclusion. The lung changes, which are of bacterial origin, and the bladder parasites (*Trichosomoides crassicauda*) occur normally in this rat colony. From a histopathological standpoint the tissues were essentially negative as far as consistent changes due to a dietary toxicant are concerned.

TABLE V

EFFECT OF DIET ON THE WEIGHT OF VARIOUS ORGANS
Mean tissue weight in mg./100 g. of rat

Diet	Number	Liver	Left kidney	Heart	Spleen
Replicate A—					
I	7	35·4	3·94	3·79	3·26
II	3	38·4	4·07	3·73	3·20
III	7	33·2	3·87	3·46	3·19
IV	11	36·0	3·76	3·54	2·71
V	11	35·7	4·05	3·65	2·85
VI	10	31·7	4·13	3·59	2·84
VII	7	36·3	3·56	3·39	2·69
Overall standard deviation		4·1	0·39	0·33	0·55
Replicate B—					
I	9	27·3	3·66	3·39	3·04
II	10	32·9	3·86	3·62	2·71
III	8	34·3	4·21	3·53	2·94
IV	10	30·0	3·48	3·49	2·68
V	10	32·4	3·83	3·65	2·62
VI	8	32·2	3·91	3·60	2·45
VII	12	30·9	3·56	3·73	2·74
Overall standard deviation		4·5	0·40	0·37	0·45

The mortality data also are presented in Table IV. Those deaths for which no explanation is given occurred at night and autolysis was too far advanced to permit a valid diagnosis. As in the previous study[1], it is confidently believed that most of these deaths may be attributed to respiratory infection. The high mortality in group II A appears to have been due to a particularly severe outbreak of respiratory infection in this cage. It is possible, of course, that the high level of all 4 potential toxicants in diet II predisposed these rats to infection, but the lack of abnormally high mortality in the corresponding group II B argues against such a possibility. In general, it may be concluded that the variations in mortality were of no significance in so far as they might reflect toxicity of the diet.

The mean weights of the livers, left kidneys, hearts and spleens of the rats surviving until killed after 32 weeks on the diets are presented in Table V. For statistical analysis the data summarised in Table V were transformed into percentage of the mean weight for each tissue and analysed by Snedecor's methods for analysis of variance with disproportionate subclass numbers[7]. The results of these analyses are presented in Table VI. Since the diet X replicate interaction was significant in one instance, and was of appreciable magnitude in the other 3 cases, these interactions were used in the calculation of F for diet and replicate. On

this basis spleen weights were significantly different (P = 0·05) between replicates. No explanation of this finding can be offered: it is suggested that this may be one of the 5 out of 100 cases where the difference is due to chance.

TABLE VI

ANALYSIS OF VARIANCE ON TISSUE WEIGHT DATA

Main effect	D.F.	Liver		Left kidney		Heart		Spleen	
		M.S.	F.	M.S.	F.	M.S.	F.	M.S.	F.
Replicates ..	1	2,005	3·6	252	1·1	250	1·1	3,000	6·7*
Diet	5	314	< 1·0	470	2·1	102	< 1·0	742	1·7
Diet X replicates	5	560	4·1*	221	2·1	229	2·5	447	1·8
Error	111	137	—	107	—	90	—	249	—

* Significant at P = 0·05.

DISCUSSION

The transient growth-depressing effect of a high level of sodium propionate in the diet of young rats in this experiment is contrasted with the lack of influence of this compound in the previous study[1]. As was noted at the time, baking the bread and later drying it resulted in a strong odour of propionate and it is probable that the final concentration of propionate in the diets of the previous study was considerably reduced. Since the present diets were unheated, little or no propionate was lost and the material at this higher level in the diet exerted an early growth-depressing effect. Obviously since normal rates of growth were resumed after a few weeks and no pathology attributable to propionate poisoning was detected, this effect of propionate cannot be regarded as too serious. In the human dietary, propionate is used almost entirely in baked goods and it is likely that the baking process would materially reduce any potential hazard.

In agreement with the previous findings, the high levels of the antioxidants butylated hydroxyanisole and propyl gallate, of the emulsifier polyoxyethylene (8) monostearate and of the flour improver chlorine dioxide had no detectable deleterious effect on the growth of rats over a 32-week feeding period. In spite of the change in experimental method, 11·25 per cent. of polyoxyethylene (8) monostearate in the diet failed to exert deleterious effects.

SUMMARY

1. Bread ingredients containing 50 times the normal concentration of chlorine dioxide, propyl gallate and butylated hydroxyanisole, or polyoxyethylene (8) monostearate given as 75 per cent. of the diet for 32 weeks did not harmfully affect growth or mortality of male rats. Sodium propionate at the same high level caused a depression of growth during the first few weeks of feeding, but did not influence mortality.

2. The high concentration of polyoxyethylene (8) monostearate, sodium propionate, antioxidants and chlorine dioxide had no detectable effect on hæmoglobin levels in the blood, on organ weights, or on histopathology of the tissues.

3. No evidence was obtained that the simultaneous presence of a high concentration of more than one potential toxicant in the diet led to any synergistic action.

The authors wish to thank Mr. Robert Van Burek and Wallace and Tiernan, Ltd., for the treatment of the flour with chlorine dioxide, the Tennessee Eastman Corporation for supplies of the antioxidant Tenox II, the J. R. Short Canadian Mills, Ltd., for a supply of Wytase, and the Lake of the Woods Milling Co. for a quantity of malt flour. They are indebted to Miss E. Connell and Mr. H. Teed for technical assistance, and to Dr. D. G. Chapman for the hæmoglobin determinations.

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...es. The present *in vivo* and *in vitro* ...s were performed using rabbit's blo... ...ne was assayed according to the m... ...oum and Gaddum as modified by Co... ...o experiments epinephrine in doses ... 0.05 to 1.0 mg/kg. was injected intrav... anesthetized rabbits. Blood sampl... ...ned from the animals by heart punc... ...nd at 10 to 15 minute intervals after ... ...n for a period of 50 to 60 minutes. I... ...es thus obtained histamine was assay... ...eucocytes were counted. For the pur... ...l similar experiments were perform... ...is which received intravenous injec... ...ological saline solution instead of epin... ...esults obtained from the in vivo exper... ...d that intravenous injection of epin... ...a decrease in blood histamine and ... ...yte count. In 37 *in vitro* experiments ...tained by heart puncture. Histami... ...d in whole blood and in plasma after ad... whole blood of equal volumes of epin... and .01 mg/10 cc) and of saline s... ...tively. The results of the in vitro ... showed that addition to whole bl... ...hrine is followed by an increase in p... ...line, presumably due to liberation of ... ...rom leucocytes. The observations de... ...ard indicate that the decrease in ...line which, under the conditions ... ...ments, followed intravenous injec... ...hrine into rabbits, was preceded by a ...amine from intracellular to extrac... ...rtments.

...elopment of seizure pattern by repe... ...al cortical stimulation. J. A. WHIELD... ...ion) and A. VAN HARREVELD. *K... of the Biological Sciences, Californ... ...nology, Pasadena, Calif.* Leão descr... spreading, transitory depression ... cortical activity which could be e... ...uli too weak to produce an afterdis... ...this spreading depression he furth... ...ed a cortical activity of a convulsiv... present experiments it was found th... ...sive activity is completely absent ... ...ression produced by the first stimul... rest (1 hour). On repetition of th... ...s at 5 to 10 minute intervals the con... ... developed and grew in intensity an... ...so it was found that a stimulus of a st... ...ration which initially was insuffic... ... an afterdischarge did so after rep... ...terdischarge and convulsive activit... ...ntensity and duration with further ... ...ere in some experiments finally fo... producing an electroencephalogram ... ...ectrically induced seizure. A rest ... ...s st...nt to restore the original si...

...stimulus produced spreading depression ...er afterdischarge or convulsive activ... ...wave of spreading depression had passed ...tex the cortical activity was abnormal ... continuous runs of medium to high ... 100-500 micro volts; slow, 2-5/sec. ...n if the stimulation produced neither ...ge nor convulsive activity.

...apacitance method for the study of cardiac WILLIAM V. WHITEHORN and EDWARD R. ...invitation). *Dept. of Physiology, Univ. ...College of Medicine, Chicago, Ill.* Stud... ...pacitative changes accompanying the ...cle in the human have continued. Sub... ...f A.C. for D.C. amplification in the ...as increased stability and gain and ...operation. Techniques and conditions ...taking have been standardized and the importance of technical factors deter... ...arther analysis of records (cardiodielec... ...in relation to simultaneously recorded ...l and mechanical cardiac events supports ...conclusions that such records are directly ...to cardiac volume changes and may provide ...mation of stroke volume. Comparisons ...ve stroke volumes calculated from cardio... ...grams of normal subjects under conditions ...exercise and performance of the Valsalva ...yield results in keeping with expected ... and provide evidence for the relative ... of the method. Absolute calibration of ...ument has been carried out by the intro... ...of spherical, saline filled balloons of known ...into the condenser field in positions ap... ...ing that of the heart. Results derived ...h calibration curves cannot, however, be ...d conclusively valid and comparison ...aneous determinations by the direct ...cedure are now in progress.

...on of dilute trypsin on muscle protoplasm ...en picture). FLOYD J. WIERCINSKI and ... A. COOKSON (introduced by JOHN C. ... *Dept. of Physiology, Hahnemann Medical ...and Dept. of Zoology, Univ. of Pennsyl... Philadelphia, Penna.* Recent studies have ...d that the colloidal behavior of muscle ...in is similar to the colloidal behavior of ...The clotting of muscle protoplasm (for ex... ...y free Ca ion) produces shortening. Inas... ...dilute trypsin solutions act like thrombin ...g blood clotting, it was thought to be of ...t to determine the effect of dilute trypsin ...cle protoplasm. The trypsin preparation ...ained 50% of a magnesium salt; the con... ...ported here are based on the trypsin ...The microinjection of a dilution of 1:1000 ...in a calcium-free Ringer solution caused ...clotting of the sarcoplasm but the sar... ...was unaffected. The interior protoplasm contracted so sharply that it broke into pieces. A somewhat weaker effect is produced by the injection of a 1:10,000 solution. The myofibrils in the interior of the fiber were seen to break in several places along the longitudinal axis. The cortical region just under the sarcolemma appeared to be unaffected by this concentration of trypsin. A 1:100,000 dilution of trypsin upon injection into the muscle fiber caused very little effect but there was a slight contraction.

Testing the validity of ashing and digestion procedures by isotope dilution. WALTER S. WILDE. *Dept. of Physiology, Tulane University, New Orleans, La.* We analyze for phosphate in the phosphoprotein-nucleic acid residue of Schmidt and Thannhauser. The sulfuric-nitric acid digest (Fiske and Subbarow, *J. Biol. Chem.* 66: 377) of intestinal mucosa from the rabbit is analyzed colorimetrically. The procedure is tested by applying the expression, $P_d^*/P_d = P_{Mg}^*/P_{Mg}$. We call the known tracer mixed with the phosphate residue before digesting P_d^*. After digestion an aliquot is neutralized and precipitated as $MgNH_4PO_4$. This is washed thoroughly, redissolved in dilute acid, and analyzed for tracer and for phosphate (colorimetric). This gives the ratio P_{Mg}^*/P_{Mg}. We then calculate for P_d which is compared to a direct colorimetric analysis of another aliquot of the acid digest. Three runs from a common pool are tabulated:

P_d^*	P_{Mg}^*	P_{Mg}	CALC.	P_d FOUND
c/sec.	*c/sec.*	*mg.*	*mg.*	*mg.*
273	22[illegible]	0.265	0.318	0.320
204	185	0.294	0.324	0.310
41	28	0.209	0.303	0.282

Although absolute loss occurs with the magnesia ppt. the tracer and non-tracer are lost proportionately so that their ratio remains unchanged and available for a valid calculation. The agreement between the value calculated and that found indicates there is no volatilization heat loss, that the presence of possible chemical interfering substances in the acid digest gives colorimetric results no different from those for the simpler solution of dissolved magnesia ppt., that pyrophosphates, if they do form, are later hydrolyzed.

Toxicity studies on antioxidants—butylated hydroxyanisole and hydroquinone. O. H. M. WILDER and H. R. KRAYBILL (introduced by A. J. CARLSON). *American Meat Inst. Foundation, Univ. of Chicago, Chicago, Ill.* Butylated hydroxyanisole (BHA) and hydroquinone (HQ) are both excellent antioxidants for use in animal fats. Toxicity studies have been made on BHA and on a mixture of BHA and HQ, using rats, to determine their LD-50s and to see if their long continued ingestion would have

any harmful effect on the animals. Six % of lard containing up to 2% of BHA was fed in a good basal ration for periods up to 21 months from weaning. No significant differences were observed in weight gains from weaning time between the controls and any of those getting the antioxidant, nor were there any differences in reproduction and growth of young. Another group of rats was started as soon after weaning as they would consume the ration and fed for 6 months at levels of BHA up to 2% of the ration. The only observed effect was a retarded weight gain at the higher levels. No effect was observed upon autopsy in any of the rats, and histological examination revealed no pathological conditions that could be attributed to the antioxidant at any level of feeding. A mixture of 80 parts BHA, 12 parts HQ, and 8 parts citric acid fed at levels of 0.3 and 2.0% of the ration has shown no effect during the growing period. The LD-50 of BHA dissolved in corn oil is 4.1 gm/kg. body weight in the rat. In a water emulsion, on a fasted rat, it is in excess of 5.0 gm/kg. body weight.

Pressure and flow of blood distal to a point of reduced arterial bore. ARNOLD H. WILLIAMS (introduced by HENRY A. SCHROEDER). *Dept. of Internal Medicine, and the Oscar Johnson Inst., Washington Univ. School of Medicine, and Barnes Hospital, St. Louis, Mo.* The decreases of pressure and flow of blood distal to a point of reduced arterial caliber are of considerable physiological importance. Heretofore studies of these hemodynamic changes, produced by external compression of an artery, have been complicated by obstruction of venous return or have been done without exclusion of collateral circulation. For this reason the changes of the pressure and flow of blood were studied in the brachial artery, distal to an adjustable clamp, in the isolated forelimb of five dogs. Blood was supplied from the animal but a perfusion reservoir was connected to the circuit to obtain pressure-flow curves. In one instance oxygen tension of the muscle was measured simultaneously. Short or prolonged arterial constrictions (up to 25 minutes) did not produce any consistent relationship between the reduction of pressure and the reduction of flow. Neither was this relationship found at the end of 15-second perfusions of the constricted artery. The calculated percentile decrease of flow was not always greater at lower perfusion pressures. Thus arterial constriction produced no consistent change of arteriolar resistance distal to the constriction.

Hepatic vital staining and dye excretion in CCl_4-treated mice. W. LANE WILLIAMS. *Dept. of Anatomy, Univ. of Minnesota, Minneapolis, Minn.* Hepatoxic centrilobular injury was produced by CCl_4 and concomitant hepatic reactions to the following were studied: *1)* two acid diazo dyes, trypan blue (Color Index 477) and chlorazol fast pink (C.I. 353); *2)* the triphe dye (also a xanthene fluoran 779); and *3)* the sulfonated trip isamine blue (C.I. 710). Dyes w taneously or intraperitoneally or aqueous solutions in amoun The azo dyes were excreted in treated mice by the kidney; fl liver; isamine blue, by liver and and isamine blue were present segregated granules. Rose Beng in macrophages in such a fashion in such cells in an observable q and isamine blue clearly staine damaged cells and remained in su ensued. The former dyes were gated into large particles durin but the latter did not clearly ind ing alteration. In contrast to Bengal stained the cytoplasm of chymal cells. At 4–6 hours afte injection normal cells were clear cells in the centrilobular area of the dye for 8–14 hours dependin (24–48 hours) subsequent to tr hepatoxic hydrocarbon.

Metabolism of the rat spinal c in isolation. R. D. TSCHIRGI (b W. GERARD, H. JENERICK (by in BOYARSKY (by invitation), J. Z. vitation). *Dept. of Physiology, Chicago, Ill.* The perfused isol lends itself admirably to combine metabolic studies. A technique h for removing the rat thoracic c vertebral bodies, fixing in plaste (after Galli-Manini) with Tyr (100 mg. %) plus purified egg alb times, washed beef erythrocy temperature, *p*H, C_{O_2}, C_{CO_2}, pressure. Flow averaged activity, tested by stimulating a recording ventral root potentials diminished in experiments lastin One minute asphyxia or 2 to 4 m without glucose abolished reflex ery was complete within 2 minu oxygen or glucose, even after more deprivation. Average glucose uti gram wet weight per hour (range 7 sponds to a Q_{O_2} of 5000. Adrena reflex response of a failing prepa flexia of aglycemia can be used to of other substrates to support fu minutes inactivity without gluco turn with glucose) the following although several are easily oxidi tate, lactate, β-hydroxybutyrate ate, succinate, adrenaline, dl ala

Fetal Resorption in the Rat as Influenced by Certain Antioxidants[1]

IRA R. TELFORD, CAROLINE S. WOODRUFF AND RAY H. LINFORD
The George Washington University, School of Medicine, Department of Anatomy, Washington, D. C.

Some prenatal mortality is perhaps a normal phenomenon accompanying gestation in all mammals. Yet there is a paucity of definitive data on total embryonic or fetal wastage as compared with the wealth of information on the pathologic, genetic, physiologic and nutritional causes of prenatal death. Of the literature which deals with this problem in man or domesticated animals, most of it deals with fetal mortality, either as stillbirths or abortions.

In the rat there are many factors that may increase fetal death and subsequent resorption as, insufficient protein (Barry, '20), elevated environmental temperature (Sundstroem, '27; Hsu, '48; MacFarlane, Pennycuik and Thrift, '57), vitamin deficiencies, e.g., biotin (Kennedy and Palmer, '45), pantothenic acid (Nelson and Evans, '46), pyridoxine (Nelson and Evans, '48), pteroylglutamic acid (Nelson, Asling and Evans, '52), tocopherol (Cheng and Thomas, '53) and riboflavin (Nelson et al., '56). Other causes of resorptions are ovariectomy (Haterius, '36), trypan blue injections (Wilson, '54), Lathyrus odoratus feeding (Walker and Wirtschafter, '56) and others. There is also evidence that loss due to resorption is not confined to any particular period of pregnancy but occurs throughout gestation (wild rat, Perry, '46; wild rabbit, Brambell, '48; mouse, Hollander and Strong, '50).

Because the rat resorbs rather than aborts an imperfect conceptus, a record of all implantations is retained in the uterine mucosa at the implantation sites. This record is represented by remnants of implantation ranging from resorbed fetuses to inconspicuous cicatricial plaques. A careful macroscopic examination of the incised term uterus will therefore supply data on total implantations as well as normal young.

Resorption data secured from the examination of nearly 500 pregnant rats involving approximately 5,000 fetuses and over 500 resorptions is herewith presented. In addition, we investigated the influence of certain maternally administered compounds, mostly antioxidants, on the incidence of fetal loss as compared to normal animals held on normal diets. These data are the outgrowth of a larger problem concerned with the possible value of these antioxidants and related compounds in the prevention or amelioration of lesions arising from neonatal anoxia.

MATERIALS AND METHODS

Walter Reed-Carworth Farms strain rats in their first gestation were used exclusively and weighed approximately 200 gm at time of breeding. After positive mating the females were randomly distributed into experimental and control groups and the former were given *per os* or directly into diet one of the various compounds listed in table 1. Twenty-two days after positive mating the pregnant rat was killed in 100% nitrogen and after 21 minutes the young delivered by caesarian section. Both horns of the uterus were completely incised and a careful macroscopic survey made for resorption sites. The resorptions were charted as to location in the respective region of the horns and classified as to degree of resorption. Seasonal variation in the number of resorptions were recorded.

RESULTS

In 126 normal untreated gravid animals 40.8% had one or more resorptions and 10.6% of all recognizable implantations had terminated in resorptions. Of the 29 dietary regimens used, animals receiving

[1] Supported by U.S.P.H.S. research grant B-1838 and United Cerebral Palsy research grant R-117j-F58.

TABLE 1

Effect of antioxidants and other compounds on fetal resorption[1]

	Total dose	Per os or in diet	Litters	Implantations	Normal fetuses	Litters with resorptions	Resorptions
	gm					%	%
Butylated hydroxytolulene (BHT)	0.5	D	11	124	121	18.2	2.4
d-l,alpha tocopherol acetate	0.2	O	12	169	162	41.7	4.1
2,5-di-tert-amylhydroquinone	0.25	D	10	134	128	60.0	4.5
d gamma tocopherol	0.2	O	11	127	121	45.5	4.7
Butylated hydroxyanisole (BHA)	0.5	D	12	145	137	51.7	5.5
d gamma tocopherol	0.07	O	12	157	148	50.0	5.7
2,6-di-tert-butyl-4-methyl-phenol (DBMP)	0.5	D	11	140	131	54.5	6.4
Thiodipropionic acid	0.5	D	10	128	119	60.0	7.0
1,2-di-hydro-2,2,4 trimethyl-6-ethoxyquinoline (Santoquin)	0.25	D	12	147	136	33.3	7.5
1,2-di-hydro-2,2,4 trimethyl-6-phenylquinoline (Santoflex B)	0.25	D	11	139	128	54.5	7.9
Nordihydroguaiaretic acid (NDGA)	0.5	D	8	86	79	75.0	8.1
Pangamic acid sodium (vit. B_{15})	0.25	D	11	138	126	27.2	8.7
Thiamine hydrochloride	2.0	O	13	160	145	69.2	9.4
Ascorbic acid	2.0	O	14	146	132	71.4	9.6
Glucose	30% of diet	D	25	280	251	68.0	10.3
Normal			126	1430	1278	40.8	10.6
Thiamine hydrochloride	0.5	D	8	75	67	62.5	10.7
2,2-methylene bis(4-methyl-6-tertiary butyl phenol)	0.25	D	8	101	90	37.5	10.9
Polymerized 1,2-dihydro-2,2,4-trimethyl quinoline (Flectol H)	0.25	D	17	148	136	52.9	12.1
Cytochrome C	0.05	D	8	84	73	50.0	13.1
Glutathione	0.25	D	6	65	56	66.6	13.9
d-l,alpha tocopherol acetate	0.1	O	14	150	129	71.4	14.0
Ascorbic acid	0.5	D	20	235	199	70.0	15.3
N,N'-diphenyl-p-phenylene-diamine (DPPD)	0.5	D	23	275	233	60.8	15.3
Phenothiazine	0.25	D	10	89	75	70.0	15.7
2,2-methylene bis(4-ethyl-6-tertiary butyl phenol)	0.25	D	9	111	92	66.6	17.1
n propyl gallate	0.5	D	9	93	76	77.7	18.3
Paraminobenzoic acid (PABA)	0.5	D	9	105	80	77.7	23.8
Hydroquinone	0.5	D	10	105	77	100.0	26.8
Methylene blue	0.5	D	10	100	62	90.0	38.0

[1] For samples of antioxidants we are indebted to the American Cyanamid Company for 2,2-methylene bis (4-methyl-6-tertiary butyl phenol) and 2,2-methylene bis(4-ethyl-6-tertiary butyl phenol), Distillation Products Industries for dl-alpha tocopherol acetate and gamma tocopherol, the B. F. Goodrich Company for DPPD, the Monsanto Chemical Company for Santoflex B, Santoquin and Flectol H and Hale Laboratories for vitamin B_{15}.

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[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Phenothiazine	0.25	D	10	89	75	70.0	15.7
2,2-methylene bis(4-ethyl-6-tertiary butyl phenol)	[illegible]	D	9	111	92	66.6	7.1
n propyl gallate	[illegible]	D	9	93	76	77.7	18.3
Paraminobenzoic acid (PABA)	0.5	D	9	105	80	77.7	23.8
Hydroquinone	0.5	D	10	105	77	100.0	26.8
Methylene blue	0.5	D	10	100	62	90.0	38.0

[1] For samples of antioxidants we are indebted to the American Cyanamid Company for 2,2-methylene bis (4-methyl-6-tertiary butyl phenol) and 2,2-methylene bis(4-ethyl-6-tertiary butyl phenol), Distillation Products Industries for dl-alpha tocopherol acetate and gamma tocopherol, the B. F. Goodrich Company for DPPD, the Monsanto Chemical Company for Santoflex B, Santoquin and Flectol H and Hale Laboratories for vitamin B_{12}.

one of 15 showed less resorptions (range 2.4–10.1%) than the controls, while rats receiving one of the other 14 diets showed an increased percentage of resorptions (10.7–38.0%) (table 1). In the dosage given none of these compounds appeared to be toxic to the mother with the possible exception of methylene blue, where poor pregnancy weight gain was noted. This compound yielded, incidentally, the highest percentage of resorptions (38.0%). Of particular interest is the observation that two of the widely used commercial antioxidants, butylated hydroxytolulene (BHT) and butylated hydroxyanisole (BHA), as well as the antioxidant vitamins alpha and gamma tocopherol, at 200 mg level, and 2,5-di-tert-amylhydroquinone gave the lowest percentage of resorptions.

Data on the distribution of resorption sites in all groups revealed that 47.6% of all resorptions were located in the cranial third of the horns, 20.6% in middle third and 31.8% in the caudal third. Most of the sites seen in this cranial region were small inconspicuous cicatrixic plaques suggestive of an early embryonic death.

Using the classification of Walker and Wirtschafter ('56) most of the resorptions, regardless of dietary supplement, belonged either to grade 3, representing complete resorption of the embryo but with small placenta still present, or grade 4, which are cicatrized uterine plaques marking previous placental sites. Grade 2, consisting of small macerated recognizable fetuses, were seldom encountered. The number of fetuses falling into grade 1, e.g., dead, but fully formed, could not be determined since many of our fetuses were killed by anoxic treatment of the mother.

DISCUSSION

Since our observations dealt with the identification of placental sites *in situ*, the question might be raised if identifiable plaques form at all implantation sites. That such scars do form where embryos have been resorbed is well documented by the work on rats of Davis and Emlen ('48). They reported more scars present than young born, the excess being ascribed to resorbed embryos. They also noted that scars do not occur spontaneously in nonpregnant rats. Moreover, these authors and Conaway ('55) reported that the resorption sites may remain identifiable for at least a year in the rat, and Deno ('41) made the same observation on the mouse. Therefore our use of first gestation animals avoided the possible error of counting sites of earlier pregnancies and ascribing them to the gestation under study.

The range of reported resorptions in normal rats by other investigators (11.8–6.5%) (table 2) may be due to seasonal variations in the fertility of the animals. It is generally accepted by rat breeders that fertility is lowest in winter and highest in summer (Main, '39). Since our data was collected throughout the year under controlled environmental conditions, we were able to separate our findings on a seasonal basis. We noted a reduction in the percentage of resorptions during the spring and summer (8.1%) and a marked increase (20.7%) during the winter and fall. Frazer ('55) using Wistar albino rats reported that 31% of all litters had some prenatal mortality on a yearly average, but that it was lowest in July-September (17%) and highest from October to December (86%). Other workers failed to present their prenatal mortality data on a seasonal basis, therefore an accurate comparison of animal groups is difficult, if not impossible.

Another variable influencing the percentage of resorptions in the rat may be the strain difference. Data on 6 different strains, including the wild rat, are summarized in table 2 and, with the exception of our WR-CF animals (10.6%) and Mason's O.M.–Mc. rats (11.8%) the 4 remaining groups agree rather closely on resorption percentages (6.6–8.7%). Yet the strain of the animal may be important, for the findings of Fekete ('47) in the mouse that fertilized ova transplanted to different strains of mice have a significantly better chance of survival in one strain than another, implies that one strain provides a better environment for implantation and development of the ovum. Hollander and Strong ('50) also noted significant differences in fetal mortality among various strains of mice. It is certainly possible that in the rat also, strain differences may exist which could

TABLE 2

Prenatal mortality in mammals

Animal	Litters	Implantations	Normal fetuses	Resorptions	Corpora lutea	Total prenatal mortality	Reference
				%			
Striped hamster	29	193	119	38.3			Fortuyn, '29
Stoat	12	108	103	4.6	135	23.7	Deanesley, '35
Grey squirrel	9		27		30	10.0	Deanesley and Parkes, '33
Sheep	80	109	101	7.2	116	12.9	Hammond, '21
Pig	22	316	267	15.5	396	32.5	Hammond, '21
Pig[1]	495	3528	3485	1.2	4480	22.2	Corner, '23
Pig	102	1117	1028	8.0	1396	26.1	Crew, '25
Rabbit (wild)	68	350	340	2.9	390	12.8	Hammond, '21
Rabbit (wild)	1819		8877		10574	16.0	Brambell, '48
Rabbit (wild)	2179	11363			12551	9.4	Brambell and Mills, '47
Rabbit (wild)	1834	9658	8927	7.6			Brambell and Mills, '48
Rabbit (tame)	56	441	391	11.3	578	32.3	Hammond, '21
Mouse	1080	7967	6672	16.3			Hollander and Strong, '50
Mouse[2]	500	3175	2930	7.7			Danforth and deAberle, '28
Rat (wild brown)	131	1238	1135	8.3	1420	20.0	Perry, '45
Rat (OM-Mc)[3]	50	492	434	11.8			Mason
Rat (Wistar)	25	226	211	6.6			MacFarlane et al., '57
Rat (Wistar)[4]	6	57	52	8.7			Wilson, '54
Rat (Long-Evans)	156		998		1498 (ova count)	33.4	Long and Evans, '22
Rat (Long-Evans)[5]	42	447	410	8.3			Nelson et al., '52
Rat (Holtzman)	35	371	347	6.5			Cheng and Thomas, '53
Rat (WR-CF)[6]	126	1430	1278	10.6			Telford, Woodruff, Linford

[1] Omitted from this data were 13 litters with all implanted embryos resorbing.
[2] From data on 500 pregnancies during last third of gestation as compared to 500 litters 1–3 days old.
[3] OM-Mc designates a mixed strain derived from Osborn-Mendel and McCollum piebald strains (data from personal communication).
[4] Reports present resorptions about 4–5% (personal communication).
[5] Pteroylglutamic acid control animals. Current resorptions in Long-Evans rats about 5% (personal communication).
[6] WR-CF indicates original Carworth Farms stock but inbred at Walter Reed Army Research Center.

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Rat (WR CF)[6]	126	1430	78	10.6		Telford, Woodruff, Telford

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[6] WR-CF indicates original Carworth Farms stock but inbred at Walter Reed Army Research Center.

account for the fairly wide range of prenatal death rates.

Our analysis of the location of resorption sites in the various regions of the uterine horn revealed that 47.6% of all resorptions were found in the cranial third of the horn, with 20.7% in middle third and 31.8% in caudal third, confirming our impression that the cephalic tapered extremity of the horn was, perhaps, an unfavorable location for the developing embryo. This concept is supported by the observation of Fawcett, Wislocki and Waldo ('47) that the overcrowding by the developing mouse ovum in the anterior chamber of the eye inhibited the growth of adjacent implanted ova. However, Frazer's ('55) data on the rat does not favor this idea, for he demonstrated that the percentage resorption was the same in both horns even though different number of ova were implanted in the different horns. Furthermore, the overcrowding of a mouse uterine horn with an unusually larger number of implanted ova did not influence adversely the resorption rate, as shown by Hollander and Strong ('50).

Another variable influencing fetal loss is the maternal age or weight. Frazer ('55) noted in two strains of rats that the heavier the mother, the higher resorption rate. Similar findings had been reported earlier by Hollander and Strong ('50). All of our animals were standardized at approximately 200 gm giving us uniform test animals thus eliminating the possible discrepancies due to age or weight.

Combining the data in table 2 for all groups of the same species, reveals that the tame rat values fall roughly in the middle of the other mammalian data. For example, the tame rat data show that 9.2% of all implantations end in resorptions. Those mammals with a lower percentage of resorptions are, pig (3.7%), stoat (4.6%), sheep (7.2%), wild rabbit (7.3%) and wild rat (8.3%). Those animals suffering on the average greater fetal loss than the rat are, tame rabbit (11.3%), mouse (15.9%) and wild hamster (38.3%). While fetal wastage is probably common to all mammals considerable species variation does exist.

Basic to our study of the value of antioxidants in the reduction of fetal mortality is the question of their mode of action and whether the site of action of the compound is on the fetal or maternal side of the placenta or both. Most of the agents reported here have been previously used in animal experimentation (Bunnell et al., '55; Schwarz, '58; Mertz and Schwarz, '58; Dam et al., '48, '51). From the results of these workers we assumed that the compound could be assimilated into the blood stream and may exert some influence on the developing placenta and might affect fetal growth. Evidence of placental transfer, however, is available only on a limited number of the tested agents, such as glucose (Huggett, '54), α-tocopherol (Neuweiler, '49), thiamine chloride (Slobody, Willner and Mestern, '49), ascorbic acid (Hamil et al., '47) and phenothiazine (Behn, Frahm and Fretwurst, '56). The rate of placental transfer of fat and water soluble vitamins is quite different, with the lipid soluble vitamins being much slower. In fact in the rat the transfer may be so slow that the cord blood content of vitamin A (Lund and Kimble, '43) or E (Straumfjord and Quaife, '46) may, under certain circumstances, be less than in maternal blood. In the rat Mason and Bryan ('40) reported only limited placental transfer of vitamin E derived from a wheat germ oil concentrate. Yet if 250 mg tocopherol is administered to women 4–8 hours before term, the vitamin E level in the cord is raised significantly (Varangot, Chailley and Rieux, '44). By contrast the water soluble vitamins, thiamine chloride (Slobody, Benson and Mestern, '46) and ascorbic acid (Manahan and Eastman, '38; Chow and Okuda, '60) showed consistently higher concentrations in cord than maternal blood, suggesting even perhaps a concentration of the vitamins by the placenta.

There is a great deal of evidence that in the rat vitamin E is essential for normal fetal development, for example, Cheng, Chang and Bairnson ('57) showed that in vitamin E deficiency 100% resorption of embryos occurred, while no resorption occurred in pregnant rats receiving daily 2 mg d,l-α-tocopherol acetate. Our data showed only 4.1% resorption in rats receiving 10 mg *per os* for 20 days during gestation as compared to 10.6% for all

normal untreated controls. Daily 5 mg dosages of α-tocopherol administered to another group of pregnant animals had no beneficial effect on resorption rate, in fact some negative response was noted (14% resorptions).

In addition to high dosages of α- and γ-tocopherol our data suggest some beneficial effects on resorption rate were obtained from 2,5-di-tert-amylhydroquinone, butylated hydroxytolulene (BHT), butylated hydroxyanisole (BHA), 2,6-di-tert-butyl-4-methylphenol (DBMP), thiodipropionic acid, 1,2-di-hydro-2,2,4-trimethyl-6-ethoxyquinoline (Santoquin), 6-phenyl-1, 2-dihydro-2,2,4-trimethylquinoline (Santoflex B), nordihydroguaiaretic acid (NDGA); while methylene blue, hydroquinone, paraminobenzoic acid (PABA), n propyl gallate, 2,2-methylene bis (4-ethyl-6-tertiary butyl phenol), phenothiazine and N, N-diphenyl-p-phenylenediamine (DPPD) all substantially increase resorption rates.

One is impressed from the data summarized in table 2 that the total prenatal mortality in mammals is much higher than might be suspected from the resorption data alone. Brambell ('48) contends in the rabbit, "that the loss between ovulation and full term is not less than 43% of ova ovulated and m be, and indeed probably is, substantially heavier." Data on total wastage in the rat is scanty, limited to the early work of Long and Evans ('22) and later Perry ('45) on the wild rat. These workers reported respectively 33.4% and 20.0% total prenatal mortality while resorption rate was 8.3% for the two strains of rats.

Other mammals follow a similar trend, ranging from an elevenfold increase in total mortality over resorptions in pig (Corner, '23) to less than a twofold increase in sheep (Hammond, '21). Such observations certainly provide a challenge to animal research workers to seek out the causative factors for such an enormous wastage. These factors are legion, but include such environmental disturbances as abnormalities of blood supply of placenta, an unfavorable implantation site, faulty development of the decidua, abnormal environmental temperature and others. Maternal influences may adversely affect normal implantation and development, such as, inanition, avitaminoses, blood dyscrasias, endocrine disorders, infections and the presence of unphysiologic compounds in the blood such as some of the antioxidants herein reported.

SUMMARY AND CONCLUSIONS

1. In 126 untreated normal pregnant rats 40.8% had one or more resorptions and 10.6% of all implantations terminated in resorptions.

2. The cranial third of the uterine horn contained 47.6% of all resorptions, while the middle third held only 20.6% of the total resorptions.

3. The percentage resorptions in the tame rat falls intermediate among the other mammalian groups.

4. Of the maternally administered compounds, the tocopherols (α and γ), BHT, BHA, amylhydroquinone and DBMP appear to have definite prophylactic value in reducing the frequency of resorptions in the rat, while methylene blue, hydroquinone and paraminobenzoic acid markedly increase resorption rates.

5. Possible extrinsic and intrinsic factors that may influence prenatal mortality in the rat are discussed.

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CHRONIC TOXICITY OF BREAD ADDITIVES TO RATS

By W. Donald Graham, H. Teed and H. C. Grice

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Currently there is much interest in the use of "chemical" additives in the food industry. Probably a considerable proportion of this interest stems from the report of Sir Edward Mellanby in 1946[1] that running fits had been induced in dogs by including in their diets flour that had been bleached and improved by treatment with nitrogen trichloride. The offending agent was finally isolated and found to be a derivative of methionine, methionine sulphoximine.[2] As a result of these discoveries several countries, Canada included, switched to the use of chlorine dioxide as a flour improving and bleaching agent. More recently, a great many compounds have been investigated in an effort to establish whether they might be added to food with a reasonable surety that no harm would result. At the time this investigation was planned a great deal of interest was being shown in the additives used in bread manufacture. Of the many compounds intentionally added to bread, chlorine dioxide, sodium propionate, the antioxidants *n*-propyl gallate and butylated hydroxyanisole, and the emulsifier polyoxyethylene (8) monostearate* were selected for study. The number of compounds which could be tested in a chronic toxicity trial was limited by the facilities available and it was considered that these 4 additives were most deserving of study.

Most investigations of this nature have been made by adding such compounds singly, in abnormally large amounts, to otherwise normal experimental diets. This may result in unequal concentrations of proteins, vitamins, minerals, and calories among the diets and the difficulty of providing adequate controls under these conditions is obvious. It appeared that a sounder method of checking for chronic toxicity would be to incorporate the additives in bread at abnormally high levels and, maintaining the levels of the nutritive elements as close to those of the control diets as possible, to give this bread as a major part of a good experimental diet. It was decided to reverse the usual procedure and to check the toxicity of the additives in combination rather than singly since the toxicity of the finished bread was of major concern.

In view of the popular "100-fold margin of safety"[3] or "margin of uncertainty" as it is rather aptly termed by Oser[1], it was decided to incorporate the additives under test in bread at 50 times the normal concentration and to give the finished bread as 75 per cent. of the experimental diet. It was considered that giving this amount would correspond to over 100 times the usual rate of intake for man.

The seven diets used are shown in Table I. The emulsifier, polyoxyethylene (8) monostearate, and the mould inhibitor, sodium propionate,

* The use of this material in bread had not been prohibited in Canada at the time these investigations were started.

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were taken as approximately equivalent, on a caloric basis, to carbohydrate and additions of these ingredients were made at the expense of glucose. On this basis all 6 bread diets were approximately isocalorific and were as near alike in all respects, except for the additives under test, as could be achieved.

Diet I contained bread made to an essentially normal formula with the exception that the glucose content was elevated to permit substitution of high levels of emulsifier and mould inhibitor in other diets. Diet II contained all 4 test ingredients at 50 times the level used in diet I. With the flour and antioxidants this was accomplished conveniently with little weight change in the diet ingredients. Diets III, IV, V and VI were similar to diet II with the exception that one ingredient in each, emulsifier, propionate, antioxidants, or chlorine dioxide, respectively, was reduced to the control concentration. Diet VII consisted of a commercial cubed diet* ground to a consistency similar to that of the other diets. The basal diet used as 25 per cent. of diets I to VI supplied adequate supplementary amounts of all the required vitamins as well as high quality protein, bulk, minerals, and fat.

Technical difficulties were encountered in the preparation of the various breads, which are referred to here by the same numbers as the corresponding diets. Those mixes which contained the high concentration of propionate showed very poor yeast action. Increasing the quantity of yeast had little beneficial effect. To overcome this difficulty to some extent the dry propionate was incorporated only into the surface of the dough so that fair yeast action in the bulk of the material was obtained. With this modification of the mixing scheme it was possible to produce breads which had been proofed approximately equivalent lengths of time. As might be expected, loaf volume and texture on numbers II, III, V and VI were poor. To a lesser extent bread I was inferior to normal quality because of the inhibitory effect of the high glucose content on yeast action. Bread IV was excessively friable and open textured.

After baking, the loaves were wrapped and stored in the refrigerator for short periods before being thinly sliced. The slices were then placed on edge in a special drier and dried in a forced draft hot air oven at 70° C. The drying process usually took 3 to 5 hours, and, for those loaves high in propionate, was accompanied, as was the baking, by a strong odour of propionate. Subsequently the dried bread was ground in a hammer mill and stored in sealed metal containers in the refrigerator (4° C.) until required for use. This dried, ground bread was rarely kept for more than 1 month. Diets were mixed at intervals of no greater than 2 weeks and were kept in closed cans in the refrigerator except during feeding periods.

A preliminary feeding trial of 3 weeks' duration indicated that there was little difference in the acceptability of the several diets to weanling rats. None of the ingredients was acutely toxic at the concentrations under test. Accordingly, a large scale experiment was started. A group of 182 male albino rats of Wistar strain ranging in age from 27 to 41 days

* Master Fox Cubes, Toronto Elevators, Toronto.

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TABLE I

DIET COMPOSITIONS

Part A. 75 per cent. of the diet	Diet						
Ingredient	I	II	III	IV	V	VI	VII
Commercial bread flour[1]	70·5	—	—	—	—	70·5	GROUND MASTER
Bread flour 50 × ClO_2[1]	—	70·5	70·5	70·5	70·5	—	
Commercial lard[2]	1·8	—	—	—	1·8	—	
Lard 50 × Antioxidants[2]	—	1·8	1·8	1·8	—	1·8	
Sodium propionate[3]	0·1	5·0	5·0	0·1	5·0	5·0	
Polyoxyethylene (8) monostearate[4]	0·3	15·0	0·3	15·0	15·0	15·0	
Cerelose (glucose)	20·4	0·8	15·5	5·7	0·8	0·8	
Salt (NaCl)	1·8	as I	as I	as I	as I	as I	
Skim milk powder	2·2	"	"	"	"	"	
Malt flour	0·5	"	"	"	"	"	
Yeast food[5]	0·2	"	"	"	"	"	
Wytase[6]	0·9	"	"	"	"	"	
Yeast	1·3	"	"	"	"	"	
	100·0	100·0	100·0	100·0	100·0	100·0	

Part B. 25 per cent. of the diet	Diet						
Ingredient	I	II	III	IV	V	VI	FOX CUBES
Casein	60·0	as I	as I	as I	as I	as I	
Alphacel[7]	9·4	"	"	"	"	"	
Corn oil	12·8	"	"	"	"	"	
Mineral mix (a)	12·0	"	"	"	"	"	
Vitamins and excipient (b)	5·8	"	"	"	"	"	
	100·0	100·0	100·0	100·0	100·0	100·0	

(a)—Minerals—the 12·0 g. of mineral mix present in 100 g. of basal diet were made up as follows—

KCl	1·580	Fe PO_4	0·195
KH_2PO_4	4·100	$KAl(SO_4)_2 \cdot 12H_2O$	0·001
$Ca_3(PO_4)_2$	1·970	$CuSo_4 \cdot 5H_2O$	0·080
Mg SO_4	1·190	NaF	0·076
$MnSO_4 \cdot H_2O$	0·027	KI	0·001

(b)—Vitamins—the 5·8 g. of vitamins and excipient in 100 g. of basal diet were made up as follows—

Thiamin hydrochloride	0·0020	Menadione	0·0004
Riboflavin	0·0040	E concentrate*	0·0560
Calcium pantothenate	0·0080	A and D concentrate†	0·1600
Pyridoxine	0·0020	Corn oil	4·2830
Nicotinamide	0·0080		
l-Inositol	0·0800		
Choline chloride	0·4000		
Folic acid	0·0008		
p-Aminobenzoic acid	0·0040		
Liver fraction‡	0·8000		

* Vitamin E concentrate, 350 mg. of *dα*-tocopheryl acetate equivalent per g.
† Navitol, 65000 A, 13000 D per g.
‡ Wilson's Liver fraction L.

NOTES

1. Chlorine dioxide.—Canadian number one patent flour was treated with 0·3 g. of chlorine dioxide per barrel (196 lb.) to yield commercial bread flour and with 15·0 g. of chlorine dioxide per barrel to yield high ClO_2 flour.
2. Antioxidants.—Tenox II, a commercial preparation containing 20 per cent. butylated hydroxyanisole, 6 per cent. *n*-propylgallate, 4 per cent. citric acid and 70 per cent. propylene glycol was used at the rates of 0·05 per cent. in commercial lard and 2·5 per cent. in high antioxidant lard.
3. Propionate.—The commercial sodium propionate sold by Du Pont de Nemours and Co., under the trade name Mycoban was used.
4. Polyoxyethylene monostearate.—The material sold by Atlas Powder Co. as polyoxyethylene (8) monostearate under the trade name Myrj 45 was used.
5. Yeast food.—The commercial material sold under the name RKD by Standard Brands was used.
6. Wytase.—This material is sold by J. R. Short Canadian Mills Limited, and is used commercially to improve whiteness.
7. Alphacel is a commercial cellulose supplying bulk without nutritive value.

and in weight from 41 to 91 g. was divided into 7 sub-groups by arranging the rats in order of decreasing weight and assigning consecutive lots of 7 rats by random selection to the sub-groups. The 182 female rats

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aged 25 to 41 days and weighing 36 to 86 g. were grouped in a similar manner. The oldest rats of each sex had been maintained on diet VII from weaning at 22 days of age until placed on test. The 14 groups of rats were housed in cages with wire screen floors. Each cage held 1 group (26 rats). Food and water were allowed *ad libitum*. For the first 3 weeks, feed consumption and body weight were recorded twice weekly; thereafter the measurements were made once a week. Cages were moved in a regular rotation on the racks so that all spent approximately equal intervals at the 4 different levels above the floor. Mortality was recorded and the probable cause of death (autopsy) was noted for those rats which died and were found before autolysis was too far advanced.

At 13 and 26 weeks, 3 rats of each sex from each diet were chosen by random selection and killed. Tissue samples from the bladder, small intestine, spleen, stomach, pancreas, adrenal, kidney, liver, heart, lung, thyroid, brain, and either testicle or ovary were preserved in formalin-saline fixative and reserved for histopathological examination. At the 52nd week all surviving animals were killed and the heart, liver, spleen and both kidneys were weighed. In addition, the usual tissue samples were reserved for histopathological examination. Gross abnormalities which were observed were recorded during this work. It should be noted that the rats were group fed and that the food consumption figures include wastage. Every effort was made to keep wastage at a minimum and there is no reason to believe, on the basis of observations made, that any particular group wasted more food than other groups.

Results

The mean body weights, at weekly intervals, of the rats in the 14 groups are presented graphically in Figures 1A (males) and 1B (females). The mean body weights of the survivors on each diet at 52 weeks and the standard error of the means are shown for males, females, and the sexes combined in Table II.

TABLE II

Final weight of survivors at 52 weeks

Weight in grams ± standard error

	Sexes combined		Males		Females	
Diet	Mean wt.	No.	Mean wt.	No.	Mean wt.	No.
I	248 ± 8·8	16	284 ± 9·8	6	226 ± 5·9	10
II	262 ± 10·8	17	288 ± 17·9	8	238 ± 6·9	9
III	266 ± 6·7	18	290 ± 7·5	8	251 ± 6·7	10
IV	251 ± 12·5	18	303 ± 25·7	6	226 ± 5·7	12
V	260 ± 9·4	22	295 ± 10·8	10	231 ± 7·5	12
VI	278 ± 16·7	14	333 ± 12·8	7	224 ± 6·8	7
VII	244 ± 7·8	19	266 ± 11·7	8	229 ± 7·7	11

The commercial diet (VII) was approximately equivalent to the bread diets in supporting the growth of female rats. Female rats on diet III surpassed in growth those on diet VII toward the end of the experiment. For male rats, with their more rapid growth, diet VII was inferior to

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bread diet I in the earlier weeks of the experiment. At 10 weeks the mean weight of male rats on diet I was significantly greater than that of male rats on diet VII. At the end of the 52nd week this difference had disappeared and the mean body weight of male rats on diet VII was not significantly lower than that of male rats on diet I or on all 6 bread diets combined.

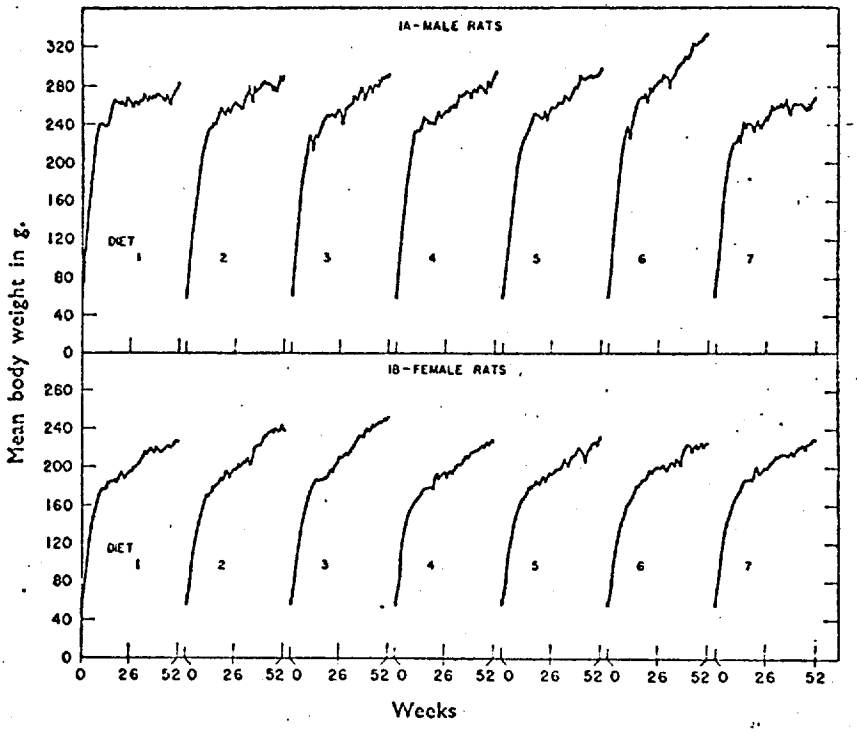

FIG. 1.

The final weights recorded in Table II were subjected to an analysis of variance based on the statistical methods of Snedecor[5] for disproportionate groups with significant interaction. The results for the commercial diet (VII) were excluded from these and subsequent calculations since the rats on this diet were included only to illustrate the usual growth, mortality, and pathology of animals from the laboratory colony. The results of the statistical analysis shown in Table III indicate that although sex had a significant effect on final weight, as was to be expected, diet was without significant influence.

The significant interaction apparently arose from the fact that male rats on the 4 diets high in polyoxyethylene (8) monostearate had a higher mean weight than those on the 2 diets low in this ingredient and female rats on the 4 diets high in chlorine dioxide had a higher mean weight than those on the 2 diets low in chlorine dioxide. These differences however were below the 5 per cent. point level of significance when the

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"t" test was applied to the appropriate means. Since the effects of diet were not significant it may be concluded that none of the additives tested, in the amounts and manner used, had any deleterious effect on the growth of male or female rats during one year.

TABLE III

ANALYSIS OF VARIANCE OF FINAL WEIGHT DATA

Main effect	D.F.	Mean square	F
Sex	1	109908	47**
Diet	5	951	<1
Diet × sex ..	5	2346	2·56*
Error	93	919	

* significant at P = 0·05 ** significant at P = 0·01

Cumulative food consumption per rat per day over the 52-week period is shown in Figure 2A. It is readily observed that the commercial diet was eaten to a greater extent than the other diets, particularly in the case of the male rats, where significantly larger amounts of the diet VII were consumed. The cumulative food efficiency data, shown in Figure 2B, were plotted in a log relationship with time and straight lines were fitted

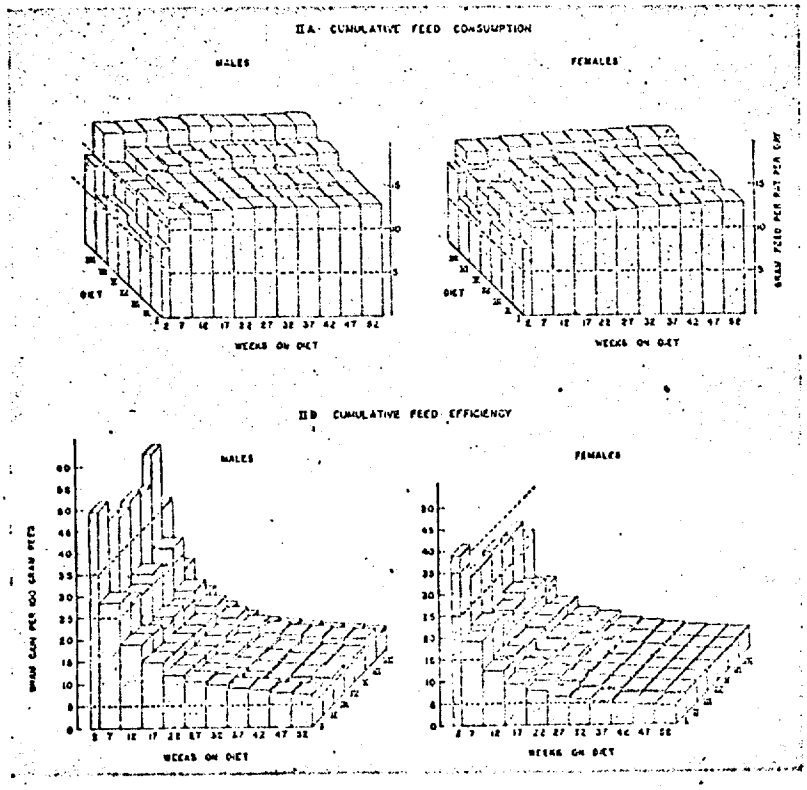

FIG. 2.

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TABLE IV

A. HISTOPATHOLOGICAL FINDINGS

	Sex													
	Male							Female						
Diet	I	II	III	IV	V	VI	VII	I	II	III	IV	V	VI	VII
Number of rats examined*	12	14	14	12	16	13	14	16	15	16	18	18	13	17
Normal rats	9	7	10	9	11	8	10	13	7	8	10	14	9	14
Brain liquefaction necrosis (embolus)	—	—	—	—	—	—	—	—	—	—	—	1	—	—
Brain inflammation (cerebromeningitis)	—	—	—	—	—	1	—	—	—	—	—	—	—	—
Lungs—respiratory infection	1	2	2	1	1	1	2	2	—	3	1	—	2	2
Heart—fatty degeneration	—	—	—	—	—	—	1	—	—	—	—	—	—	—
Liver—cloudy swelling, fatty degeneration	—	1	—	—	—	1	1	—	2	—	—	—	—	—
Liver—excessive deposits of haemosiderin	—	—	—	—	—	—	—	—	—	—	2	—	—	—
Spleen—excessive deposits of haemosiderin	—	—	—	—	—	—	—	—	—	—	2	—	—	—
Stomach—inflammation of submucosa	—	—	—	—	1	—	—	—	—	—	—	—	—	—
Intestine—erosion	—	—	—	—	—	—	—	1	—	1	2	—	—	—
Kidney—degeneration of tubules	—	—	—	—	1	—	—	—	1	—	2	1	2	—
Kidney—glomerulonephritis	—	—	—	—	—	—	—	—	2	2	—	—	—	—
Kidney—hydronephrosis	—	—	—	—	—	—	—	—	1	1	—	1	—	—
Adrenal—cloudy swelling, necrosis in zona fasiculata	—	—	—	—	1	1	—	—	1	—	—	1	—	—
Bladder—parasite Trichosomoides crassicauda	2	5	2	3	3	1	1	—	1	3	—	1	2	2
Testicle—degeneration	—	—	1	—	—	—	—	—	—	—	—	—	—	—

* 3 rats were killed at 13 weeks, 3 at 26 weeks, and the remainder at 52 weeks.

B. MORTALITY DATA

Died on test	14	12	12	14	10	13	12	10	11	10	8	8	13	9
Undiagnosed	5	4	9	7	6	9	8	7	9	5	4	7	8	4
Respiratory infection	8	6	3	7	4	3	3	1	2	5	4	1	2	5
Middle ear disease	1	2	—	—	—	1	1	2	—	—	—	—	1	—
Tumor—fibroadenoma	—	—	—	—	—	—	—	—	—	—	—	—	2	—

to the points by the method of least squares. These lines were not significantly different for female rats but for the males the food efficiency on diet VII was significantly less than that on diet I.

The sections from the tissues of the 208 rats were stained with hæmatoxylin and eosin and examined microscopically. The group to which the various rats belonged was unknown to the pathologist (H.C.G.) at the time the tissues were examined. The histopathological findings are presented in Table IVA and the results have been summarised by the pathologist as follows:

"The tubular degeneration and perhaps the glomerulonephritis seen mainly in the kidneys of female rats are the only histopathological findings considered to be significant. The tubular degeneration is, in all probability, due to a toxic substance. The lack of coexisting glomerular change is remarkable as the glomerulus is usually affected before the tubules. It may be that there is a tubular specificity of the injurious agent or that there is an inherent weakness of the tubular epithelium in these particular rats. The former supposition is more probable. The hydronephrosis in each case was unilateral. Dilatation of the kidney pelvis may be caused by concretions, parasites, or tumors in the urinary bladder or it may be congenital. Concretions were not found in any of the bladders, there were no tumors present in the bladders of any of the animals examined, and there was no evidence of congenital atresia of the ureters. While parasites were not observed in the bladders of rats suffering from hydronephrosis, these could have been missed in histological

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cross section (4 microns) and the known incidence of such parasites is sufficient to attribute the hydronephrosis to bladder parasites."

The significant kidney changes in female rats were found on diets II, III, IV and V. While the incidence is low, it should be noted that these were the rats receiving flour treated with the high concentration of chlorine dioxide. Tubular damage from propyl gallate has been demonstrated by Orten, Kuyper and Smith[6], and this may have been a contributing factor. Again, the incidence is so low that the distribution may be fortuitous.

The overall mortality data are tabulated in Table IVB. It will be noted that a considerable number of deaths occurred for which no explanation is given. These deaths occurred at night and by the time the bodies

TABLE V

EFFECT OF DIET ON THE WEIGHT OF VARIOUS ORGANS
Mean tissue weights in mg. per g. of rat

Diet	Number	Liver	Right kidney	Left kidney	Heart	Spleen
A. Males						
I	6	32·7	3·56	3·55	3·96	2·82
II	8	31·0	3·66	3·83	3·81	2·51
III	8	32·5	3·72	3·71	3·65	2·71
IV	6	37·4	3·38	3·31	3·22	2·16
V	10	34·0	3·60	3·51	3·42	2·64
VI	7	34·2	3·47	3·35	3·38	2·59
VII	8	34·1	3·41	3·19	3·84	2·67
Over-all standard deviation		4·6	0·43	0·42	0·40	0·38
B. Females						
I	10	35·0	4·10	4·07	4·48	3·47
II	9	39·5	4·95	5·06	4·29	3·31
III	10	41·5	4·82	4·81	4·70	4·01
IV	12	37·3	4·28	4·24	4·10	4·03
V	12	38·0	5·19	5·02	4·68	3·65
VI	7	35·6	4·46	4·61	4·47	3·43
VII	11	38·4	4·02	4·06	4·08	3·59
Over-all standard deviation		4·5	0·81	0·67	0·42	0·85

TABLE VI

ANALYSES OF DATA ON TISSUE WEIGHTS

Main effect	D.F.	Liver		Right kidney		Left kidney		Heart		Spleen	
		m.s.	F.	m.s.	F.	m.s.	F.	m.s.	F.	m.s.	F.
Sex	1	3088	15*	15932	49**	16934	61**	11508	46**	25473	33**
Diet	5	212	1·0	618	2·1	714	2·6	299	1·2	361	<1·0
Sex × diet ..	5	203	1·4	324	<1·0	276	2·1	248	3·4**	765	1·5
Error	93	147		397		133		73		493	

* significant at P = 0·05 ** significant at P = 0·01

were retrieved in the morning autolysis was too far advanced to allow conclusive autopsy findings. It may be stated with a considerable degree of confidence that most of these deaths were due to respiratory infection. A χ^2 analysis of the data revealed no significant influence of diet on mortality.

The mean weights of the livers, kidneys, hearts, and spleens of the rats which survived to the time of sacrifice at 52 weeks are listed in Table V.

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For statistical analysis the data in Table V were transformed into percentages of the mean weight for each tissue. The resulting data were analysed by Snedecor's methods for disproportionate subclass numbers. The results of these analyses are presented in Table VI.

As might be expected, sex had a significant influence on the weight of the tissues examined. Since the sex × diet interaction was significant in one instance and in 4 of 5 instances the mean square for interaction was of appreciable magnitude these interactions were used in the calculation of the F for diet and sex. In no instance did diet have a significant influence on tissue weight.

Discussion

Propionate.

Very few experimental data on the toxicity of sodium propionate are available. In a 3-week trial Harshbarger[7] fed concentrations of 1 and 3 per cent. sodium or calcium propionate in the diets of rats. No effect on weight gains was observed. Heseltine[8] reported that sodium propionate had a weak antihistamine action about 1/7·5 that of diphenhydramine. Daily oral doses of 6 g. to men led to the production of a fairly alkaline urine but no appreciable diuresis, catharsis, or other effects.

These findings are confirmed and extended by the present data which show that at concentrations of 5 per cent. in bread ingredients before baking, a finished product is obtained which exerts no discernible toxicity when fed as 3/4 of the diet of rats for 1 year; this in spite of the added stress of the presence of 3 other additives at high concentration in the diet.

Antioxidants.

n-Propyl gallate is a fat-soluble ester of the widely-occurring natural antioxidant gallic acid. It has been fairly intensively investigated by Orten *et al.*[6], who found that concentrations of 1·17 per cent. in the diet of male rats for 6 months caused growth depression, a decrease in hæmoglobin levels, and some damage to the kidney tubules. Guinea-pigs receiving 0·011 per cent. in the diet for 1 year and dogs receiving similar amounts for 14 months showed no ill-effects. Lehman, Fitzhugh, Nelson and Woodard[9] found this antioxidant even less toxic and, in rats, detected no growth inhibition at 1 per cent. of the diet of males or females. At 5 per cent. of the diet growth depression occurred in male rats, there was some increase in mortality over the 2-year feeding period, and there was evidence of patchy hyperplasia of the proventriculus. Allen and De Eds[10] and Van Sluis[11] reported very low toxicity for lauryl gallate given to rats.

Wilder and Kraybill[12] reported that butylated hydroxyanisole (the commercial material is usually a mixture of the 2- and 3-tertiary butyl esters) when given to rats for periods up to 21 months at concentrations up to 0·06 per cent. of the ration caused no toxic effects. At 0·12 per cent. palatability of the ration was lowered but no pathology was detected. Evidence accumulated by the U.S. Food and Drug scientists[13] indicated that this antioxidant was safe when used in the usual concentrations.

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The amounts of the several ingredients of the antioxidant mixture fed in the various diets is shown in Table VII.

In the amounts in which they occurred there is no reason to suspect any direct toxic action from the propylene glycol or the citric acid. The high level of butylated hydroxyanisole was about 1/10 that found to cause no harmful effects and the high level of *n*-propyl gallate was about 1/50 of the maximum non-harmful amount. Even the combination of these 2 failed to exert any significant influence on the health of the rats.

TABLE VII

ANTIOXIDANT AND PROPYLENE GLYCOL CONTENT OF DIETS I–VI

	Diets I and V	Diets II, III, IV and VI
	mg./kg.	mg./kg.
Butylated hydroxyanisole ..	1·35	67·50
n-Propyl gallate	0·405	20·25
Citric acid	0·27	13·50
Propylene glycol carrier ..	4·725	236·25

Polyoxyethylene (8) *Monostearate.*

The literature on the toxicity of polyoxyethylene (8) monostearate is rather considerable. The chief conclusion reached after a careful scrutiny of these data is that, under certain conditions, polyoxyethylene (8) monostearate in fairly large amounts in the diet may cause harmful effects[14,15,16,17]. Whether or not these certain conditions are of such a nature as to lend practical significance to the observed toxicity is a matter of argument. The literature is too voluminous to review in detail here. Suffice to say that loss in weight, diarrhœa, bladder stones, etc., have been observed in rats fed diets containing 5 per cent. or more of this emulsifier. Other investigators have not demonstrated any harmful effects at concentrations up to 5 per cent. in the diet[18]. To quote Frazer[19] "Grossly excessive amounts of polyoxyethylene monostearate—some workers have used as much as 15 per cent. of the diet—may produce some effects. Some of the results of these experiments are not convincing and in any case they have little relevance to the normal dietary use of these materials."

Culver, Wilcox, Jones and Rose[20] have shown that in humans receiving polyoxyethylene (40) monostearate the polyoxyethylene was recovered almost completely in the urine and fæces, that this portion of the molecule was recovered free of combined fatty acid, and that there was no evidence of storage.

In the experiments reported here, polyoxyethylene (8) monostearate occurred as 0·3 or 15·0 per cent. of the breads which made up 75 per cent. of the diet. Under these conditions and in spite of 2 or 3 additional dietary stresses, no toxic effect of this material was observed.

Chlorine Dioxide.

The toxicity of chlorine dioxide-treated flour has been the subject of several investigations. Arnold[21] gave dogs, as 80 per cent. of their diet, flour treated with 5 g. of chlorine dioxide per 100 lb. for periods up to 28 days without detecting any ill-effect. Newell, Gershoff, Suckle,

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Gilson, Erikson and Elvehjem[22], carried out similar test giving 60 per cent. flour in the ration with a maximum chlorine dioxide treatment of 4 g. per 100 lb. and continuing the trials for 13 weeks. The results of these studies on dogs were negative. Similar studies on rabbits (6 weeks) monkeys (5·5 months) and rats (5 weeks) also gave negative results. A group of 13 humans receiving 55 g. of chlorine dioxide-treated wheat gluten (20 g. of ClO_2 per 100 lb. of gluten) daily for 6 weeks showed no detectable injury. Nakamura and Morriss[23] gave to dogs flour treated with high concentrations of chlorine dioxide for prolonged periods without seeing any evidence of canine hysteria. With the exception of the last experiment most of the studies were of short duration and no long-term studies on rats have been reported.

In the present investigation it has been shown that rats receiving a diet containing 75 per cent. of bread made from flour treated with 15 g. of chlorine dioxide per barrel (196 lb.), 50 times the normal rate of treatment, for 1 year from weaning age exhibited no signs of toxic effect as evidenced by growth, mortality, gross observation, organ weights or histopathological examination of the tissues.

Summary

1. Bread containing 50 times the normal concentration of chlorine dioxide, propyl gallate and butylated hydroxyanisole, polyoxyethylene (8) monostearate, or sodium propionate as 75 per cent. of the diet for 1 year did not harmfully affect growth or mortality of rats.

2. The high concentrations of polyoxyethylene (8) monostearate, sodium propionate, antioxidants and chlorine dioxide had no detectable effect on organ weights or on histopathology of the tissues.

3. No evidence was obtained that the simultaneous presence of high concentrations of more than one potential toxicant in the diet led to any synergistic action.

The authors wish to thank Mr. Robert Van Burek and Wallace and Tiernan, Ltd., for the treatment of the flour with chlorine dioxide, the Tennessee Eastman Corporation for supplies of the antioxidant Tenox II, the J. R. Short Canadian Mills, Ltd., for a supply of Wytase, and the Lake of the Woods Milling Co. for a quantity of malt flour. They are deeply indebted to Miss E. Connell for technical assistance in the histopathological work. The laborious tasks of mixing and baking the experimental breads was carried out by Miss Margaret Lock and Miss Dorothea Tripp of the Nutrition Division of this Department. The authors express their gratitude for this assistance and for the interest and advice of Dr. L. B. Pett, Dr. L. I. Pugsley, Mr. M. G. Allmark, and Dr. J. A. Campbell of this Department and of Mr. N. Gridgeman of the National Research Council.

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Ovicidal Effect of Selected Compounds on the Eggs of *Anopheles albimanus*[1,2]

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ABSTRACT

Effectiveness of selected compounds as ovicides was investigated under laboratory conditions against melanized and unmelanized eggs of *Anopheles albimanus* Wiedemann. In most cases, unmelanized eggs were more susceptible to the toxicants tested. Melanization was necessary for egg hatch. Conversion of tyrosine to dihydroxyphenylalanine (DOPA) by tyrosine hydroxylase is the slowest enzymatic step in the overall synthesis of melanin. Tropolone, which has been reported to be an inhibitor of this enzyme, was 10 times more effective against unmelanized eggs than against melanized eggs, thus suggesting inhibition of tyrosine hydroxylase.

Structural activity relationships were established. Ovicidal efficiency increased with increase in alkyl side chain on the phenolic and polyhydric phenolic moiety. Aromatic and alicyclic substituted phenols were the most effective compounds tested as ovicides. The most potent antioxidant found was butylated hydroxyanisole (a food additive). When exposure periods to selected ovicides were less than 16 hr, mortality decreased. There was no marked increase in ovicidal effect when concentrations of ovicides were increased with an exposure period of 4 hr. The data indicate that penetration of the chorion is dependent on molecular configuration of the ovicide.

The sequence of events in embryogenesis provides opportunities for inhibition of enzymes. Conversion of tyrosine to dihydroxyphenylalanine (DOPA) by tyrosine hydroxylase is the slowest enzymatic step in the overall synthesis of melanin. This step represents a highly vulnerable link in the biosynthetic chain and also represents a logical point of attack for selective interference with melanization. Melanization is necessary for egg hatch for *Anopheles albimanus* Wiedemann. Many classes of compounds have been screened already as potential tyrosine hydroxylase inhibitors (Levitt et al. 1967, McGeer et al. 1967). Catechols are such inhibitors. In addition to enzyme inhibitors, reducing compounds (antioxidants) may function by preventing quinone from being oxidized to melanin. Compounds which complex with glutathione or disturb oxidative-reductive mechanisms may distort the pathway of enzymatically directed reactions in embryogenesis. Structural activity relationships depend not only on enzyme substrate interaction but also on penetration of the egg shell. Compounds were selected as ovicides on the basis of interference with the systems described.

Compounds which have been used most extensively as ovicides, individually and in combination, are the dinitrophenol compounds and petroleum oils (Tattersfield et al. 1925, Gimingham and Tattersfield 1927). Dinitrocyclohexylphenol (DNOCHP) was used in combination with petroleum oil for aphid scale and mite control in deciduous fruit orchards and in citrus groves (Boyce and Pendergast 1936, Dutton 1936). Use of the dinitro petroleum oil combinations decreased because of phytotoxic hazards, and once again the individual materials were applied separately. Although usefulness of the dinitro compounds has been limited by phytotoxicity, unpleasant handling properties, and application problems, they are still effective ovicides for several species of aphids. Petroleum oils exert ovicidal action by interference with the respiratory exchange of gases (Smith and Pearce 1948). Interference must continue for a critical period to prove lethal, and in the case of eggs of *Grapholitha molesta* Busck, the oriental fruit moth, this period is ca. 24 hr at 80°F. Oils which volatilize from the surface of the chorion before the critical period is over are inefficient ovicides. Dinitrophenol compounds may upset the normal process of glycolysis by uncoupling oxidative phosphorylation. The significance of the ATP/ADP ratio in the process of oxidative metabolism of insects has been determined (Chefurka 1965). Most of the homologous series of primary aliphatic amines (long alkyl chains) proved highly effective against eggs of *A. albimanus* (Mulla and Chaudhury 1968).

MATERIALS AND METHODS.—In experiments with unmelanized eggs of *A. albimanus*, 50 ♂ and 50 ♀ newly emerged mosquitoes in 1-gal cylindrical cardboard breeding cartons were placed on a diet of honey water for 3 days; then they were offered daily a blood meal in addition to honey water. Three days after the 1st blood meal, an aqueous solution of a compound in a filter-paper-rimmed oviposition dish was inserted into the carton. Compounds were dissolved in alcohol, and serial dilutions were made in this solvent before water was added to obtain the desired volume. The following morning, observations were made on the number and the nature of the eggs deposited in the oviposition dish.

Two groups of 100 eggs were removed from the oviposition dish and placed in the center of a spherical area cut out of a wax paper float. The float was placed on 25 ml of water in a petri dish. The float prevented eggs from becoming stranded on the wall of the dish above the water line. Contact with glass or plastic under these conditions causes dehydration of the eggs. Upon hatching, the larvae were counted daily for 7 days.

In experiments with melanized eggs, the same procedures were followed except that eggs were laid in untreated water. Two groups of 100 melanized eggs each were then placed in an aqueous solution of the compound for 16 hr. Upon completion of this period, the eggs were transferred to petri dishes containing water as described. The petri dishes were examined daily for larvae.

Melanized eggs were exposed to butylated hydroxyanisole (BHA) at a concentration of 200 ppm for 2, 4, 6, 8, 12, 14, and 16 hr. Melanized eggs were treated also with 25, 50, and 75 ppm of cinnamyl alcohol. Contact time with this ovicide was 4, 8, 12,

[1] Diptera: Culicidae.
[2] Use of trade names is for identification purposes only and does not constitute endorsement by the U.S. Public Health Service.
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Table 1.—Ovicidal action of phenolic compounds against eggs of *A. albimanus*.

Compound	Melanized eggs		Unmelanized eggs	
	Concn (ppm)	% hatch[a]	Concn (ppm)	% hatch[a]
Phenol	200	46	200	17
m-Cresol	100	40	100	35
p-Ethylphenol	50	37	33	3
p-tert-Butylphenol	50	67	50	58
p-tert-Amylphenol	50	74	50	11
Cyclohexylphenol	16	6	10	34
4-Chloro-2-cyclopentylphenol	10	4	5	5
Tropolone	25	29	2.5	34
4-*tert*-Butyl-2-chloro-phenol	25	69	10	50
m-Chlorophenol	100	3	33	74
Aromatic substituted				
o-Phenylphenol	5	60	5	58
p-Phenylphenol	5	5	5	36
m-Phenoxyphenol	5	3	5	11
2-Chloro-6-phenylphenol	2.5	36	1	27

[a] Control hatch 90% or above.

and 16 hr. Each treatment was run in duplicate. At the end of each exposure period, eggs were transferred to petri dishes containing water and the larvae were counted as described. Experiments with ovicides were carried out at 80°F and 50% RH.

RESULTS AND DISCUSSION.—A search for more effective control of insects can hardly be considered complete without consideration of the use and action of ovicides. To obtain a more comprehensive picture of the ovicidal effect of the compounds tested, melanized and unmelanized eggs of *A. albimanus* were used in the experiments. The present investigation studied phenolic compounds without the nitro substituent and other related compounds. Some are highly effective ovicides and are practically nontoxic to mammals, since they are used as food additives, germicides, and antimicrobials for fruits and vegetables.

In experiments with the unmelanized eggs, color was noted after eggs were deposited overnight in an aqueous solution of the ovicide. Some were straw colored, indicating melanization had not occurred and that the embryo had died. Other eggs were darker in hue, indicating that embryos had developed further, perhaps almost to the point of hatching. However, presumably weakened by the toxicant, they were unable to break through the chorion. Later examination showed some dead larvae partially out of the egg shell. These eggs also were counted as unhatched. Three compounds tested produced this latter effect: *p*-ethylphenol, *p-tert*-butylphenol, and *p-tert*-amylphenol. A good ovicide does not necessarily have to prevent all eggs from hatching provided the larvae that hatch from the remaining eggs die immediately following emergence.

Table 1 shows the substituted phenols evaluated as ovicides against melanized and unmelanized eggs of *A. albimanus*. Several concentrations of each compound were tested. Those that resulted in either no mortality or 100% mortality are not included in the table. Such extremes in mortality are not suitable for comparison of the compounds tested. Data presented are based on a minimum of 2 replications/dosage. The presence of an alkyl group on the phenolic ring increases effectiveness as an ovicide. Cresol (*o*-methylphenol) is twice as effective as phenol. Effectiveness increases 4-fold with *p*-ethylphenol. Since *p-tert*-butylphenol and *p-tert*-amylphenol are not more effective as ovicides than *p*-ethylphenol, the toxicity apparently increases with increasing chain length to 2 carbon atoms. Tattersfield et al. (1925) first investigated the structure vs. activity relationship of the nitrophenols to *Aphis rumicis* L. Their studies showed that toxicity was associated with a nitro group para to the hydroxyl group, and that maximal activity occurred with 2,4-dinitrophenol alkylated in the ortho position as in 2,4-dinitro-6-methylphenol. Kagy (1941) extended this series to include effects of various ortho-alkyl side chains, and his studies showed that toxicity increased with increasing chain length up to *n*-heptyl and then decreased. An increase in chain length may facilitate penetration of the egg shell. Steric effects of the alkyl group may have a profound effect on overlap of the enzyme surface preventing enzyme substrate interactions. Alicyclic-substituted phenols are more effective ovicides than the alkyl substituted phenols for both melanized and unmelanized eggs (Table 1). Structural requirements for penetration of the egg shell and enzyme substrate interactions are perhaps more closely met with the alicyclic than the alkyl substituted compounds. The chlorine group on the phenolic moiety increases effectiveness of the ovicide, since 4-chloro-2-cyclopentylphenol is 1.6 times more effective than cyclohexylphenol against melanized eggs. Increase in effectiveness by the chlorine group is further confirmed in that chlorophenol is more effective than phenol. Also, the chlorinated compounds show their greatest effect against unmelanized eggs. The chlorine group has an inductive or electron withdrawing effect, thus the acidity of chlorophenol is greater than that of phenol. The acidity of the molecule may play a role in its function. Alicyclic substituted compounds are more effective toward the unmelanized than melanized eggs (Table 1). Differences in permeability no doubt account for this phenomenon.

Aryl-substituted phenols were the most effective ovicides. The steric effect of the aryl group is greater than the alkyl and alicyclic substituent. The aryl-substituted phenols as well as alicyclic - substituted phenols were difficult to evaluate because of their slight solubility in water. This difficulty was especially evident for crystalline compounds, which aggregate even in dilute aqueous solutions. A liquid, *m*-

Table 2.—Polyhydric phenols as ovicides against eggs of *A. albimanus*.

Compound	Melanized eggs		Unmelanized eggs	
	Concn (ppm)	% hatch[a]	Concn (ppm)	% hatch[a]
Catechol	1200	12	200	23
Resorcinol	2400	80	200	2
Hydroquinone	2400	46	200	6
p-tert-Butylcatechol	400	41	50	34
Hexylresorcinol	50	4	25	12
2-Nitroresorcinol	20	5	12	6

[a] Control hatch 90% or above.

phenoxyphenol, seemed to disperse better than its solid counterpart, and this fact could explain why this compound was more effective than *o*-phenyl phenol. *o*-Phenylphenol is of interest since Hodge et al. (1952) reported the low order of acute toxicity in rats, LD_{50} 2.7 g/kg. This low order of toxicity permits use of this compound in preventing postharvest microbial spoilage of fruits and vegetables. Smith (1962) reported that *o*-phenylphenol was used in concentrations ranging from 0.1 to 6% for fruits and vegetables by dipping, spraying, and hydrocooling methods. Tropolone was selected as an ovicide because it is a cycloheptanone. Isosterism between tropolone and the catechol ring suggests that tropolone might also be an inhibitor of tyrosine hydroxylase. Tropolone at 2.5 ppm prevented 50% of the eggs of *A. albimanus* from melanizing. Only 34% of the remaining melanized eggs hatched. At a concentration of 25 ppm, the compound had ovicidal activity against fully melanized eggs with a 29% hatch. This difference in effectiveness against the 2 types of eggs indicates that the target enzyme for this compound is tyrosine hydroxylase. Goldstein et al. (1967) reported that 4-isopropyl tropolone was an effective inhibitor of tyrosine hydroxylase in vivo and in vitro with adrenal medulla extracts. In the aryl series, the chlorine group also enhances ovicidal activity.

Table 2 shows the polyhydric phenols evaluated as ovicides against melanized and unmelanized eggs. The dihydroxybenzenes (catechol, resorcinol, hydroquinone) are as effective as phenol against unmelanized eggs. With melanized eggs, 6–12 times the concentration of the dihydroxybenzenes are required to produce the same effect as phenol. The presence of an alkyl group on the polyhydric phenol moiety increases ovicidal activity. Toxicity increases with increase in chain length, since hexyl resorcinol is 50 times more effective than resorcinol. The nitro group also increases ovicidal activity as demonstrated by 2-nitro resorcinol.

Table 3 shows the effectiveness of antioxidants as ovicides against unmelanized eggs. Since molecular geometry obviously determines whether an antioxidant will penetrate the chorion, closely similar antioxidants may act differently. Butylated hydroxyanisole (BHA), a food additive, was the most effective ovicide in the series tested. Closely related butylated hydroxytoluene had no ovicidal effect. This difference in ovicidal effect can be attributed to the amount of toxicant penetrating the chorion which is dependent upon the water solubility as well as structure of the compound. Cleland's reagent (2,3-dihydroxy-1,4-dithiobutane) is the antioxidant of choice in biochemical systems because of its low redox potential

Table 3.—Effectiveness of antioxidants as ovicides against unmelanized eggs of *A. albimanus*.

Compound	Concn (ppm)	% hatch[a]
Mercaptoethanol	400	3
Cleland's reagent	1000	74
Cysteine	1000	70
Glutathione	5000	81
Mercaptoethylamine	1000	83
Propyl gallate	1000	31
Butylated hydroxyanisole	50	7

[a] Control hatch 90% or above.

Table 4.—Ovicidal action of alcohols and substituted alcohols against eggs of *A. albimanus*.

	Melanized eggs		Unmelanized eggs	
Compound	Concn (ppm)	% hatch[a]	Concn (ppm)	% hatch[a]
1-Octanol	700	20	—	—
Phenylethyl alcohol	800	57	400	47
3-Phenyl-1-propanol	400	19	75	55
Cinnamyl alcohol	12	55	10	46
1-Phenyl-1-heptanol	25	11	20	23
2-Phenoxyethanol	800	23	400	47
2-(*p-tert*-Butylphenoxy)-ethanol	100	38	26	36

[a] Control hatch 90% or above.

and other convenient properties such as solubility in water and alcohol. Apparently, Cleland's reagent does not possess the configuration that is necessary for penetration through the chorion.

Table 4 shows alcohols and substituted alcohols that were effective as ovicides against melanized and unmelanized eggs of *A. albimanus*. Alcohols are reagents for extraction of lipoidal materials and may change the lipid integrity of the egg shell. 1-Octanol prevented the mosquitoes from depositing eggs. Presence of a phenolic substituent on the alcohol moiety increased ovicidal properties. With this substituent, ovicidal activity increased with increase in the chain length of the alcohol. This series of compounds was more effective against unmelanized eggs. Structural differences caused by the polymerization of indole quinone and subsequent attachment to protein can influence the amount of toxicant entering the ovum. Of the alcohols and substituted alcohols tested, cinnamyl alcohol was the most effective, killing 55% of the eggs at 12 ppm. The closely related phenylethyl alcohol was far less effective. Apparently cinnamyl alcohol has the proper configuration for penetrating the egg chorion. *N*-ethyl maleimide (NEM) was tested as an ovicide because Guntherberg and Rost (1966) showed that this compound complexed with reduced glutathione (GSH) and thus prevented its oxidation to oxidized glutathione (GSSG). This transformation may be involved in melanization. NEM was effective at 65 ppm against unmelanized eggs. At this concentration, 50% of the eggs did not melanize, but 7% of those that did melanize also hatched.

To study the effect of exposure periods on hatch of *A. albimanus* eggs, several relatively effective compounds were employed. Fig. 1 presents graphically the percentage of hatch when melanized eggs were exposed to 0.02% BHA at increasing exposure times. The percentage hatch decreased as the contact time with the ovicide increased. An exposure of 16 hr was necessary to kill all eggs. The amount of BHA penetrating the chorion is thus time dependent.

To evaluate the relationship between changes in concentration and increasing exposure, cinnamyl alcohol was chosen as the test ovicide. Fig. 2 shows the effects. It appears that increasing the concentration of cinnamyl alcohol has no effect on embryonic development during the exposure period of 4 hr. The concentration effect becomes apparent at the 8 hr exposure time. Absence of evidence of abrupt inhib-

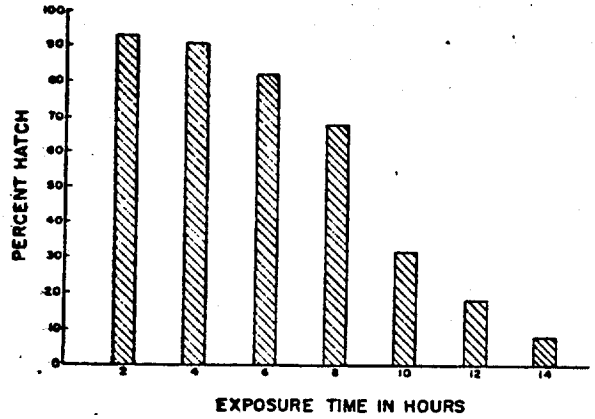

FIG. 1.—Effectiveness of 0.02% butylated hydroxyanisole in relation to time of exposure on melanized eggs of *A. albimanus*.

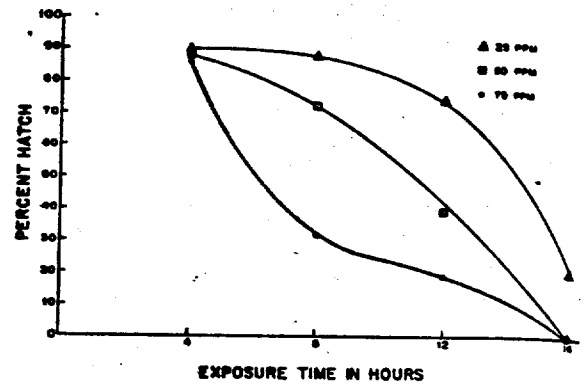

FIG. 2.—Percent hatch in relation to time of exposure and concentration with cinnamyl alcohol as ovicide.

ition of embryonic development indicates that cinnamyl alcohol and BHA act slowly on the chorion gradually diffusing into the ovum.

The relationship between ovicidal efficiency and chemical structure is complex. The antioxidants, as well as the other compounds tested, must have the proper configuration to penetrate the chorion and produce ovicidal activity. Increasing the polarity of the compound decreases its effectiveness as an ovicide. One may conclude that highly polar-hindered compounds do not penetrate the chorion, since quercitin, an antioxidant, at 200 ppm had no ovicidal effect. Since eggs are susceptible to long-chain alcohols, the lipophilicity, in addition to molecular geometry, plays a part in predicting whether a compound will penetrate the chorion.

The present data indicate that enzymes such as tyrosine hydroxylase can be inhibited. Furthermore, enzymes play a role in regulating the hatching processes. One can therefore speculate that having the proper configuration and properties, the ovicide enters the egg and inhibits a specific enzyme. Recent investigations by Van Woert (1967) demonstrated that melanin can participate as an electron acceptor in certain metabolic pathways. Their in vitro experiments indicate the ability of melanin to oxidize the coenzyme, reduced nicotinamide adenine dinucleotide (NADH). Melanin may therefore play a role in oxidative-reductive mechanisms.

Proper formulations of compounds which are highly effective against eggs of *A. albimanus* and which are innocuous to nontarget organisms may offer a potential method of control of the egg stage in the environment.

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Certain Organic Substances and Their Effects upon the Incidence of Dental Caries in the Cotton Rat

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This paper summarizes a series of experiments that was conducted from 1958 into 1964. The effects of different types of compounds on the incidence of experimental dental caries in the cotton rat are presented. The purpose of these assays was to study the influence of a number of discrete organic compounds on the incidence and severity of experimental caries in the cotton rat and to obtain information as to the nature of the fractions responsible for the cariostatic activity of oat hulls.[1]

TABLE 1

EFFECT OF CERTAIN AMINO ACIDS ON DENTAL CARIES

Compound	Conc. in Diet (g/kilo)	No. of Animals	Av. Gain (gm.)	Caries Incidence	Per Cent Reduction*
Cariogenic control†	.....	90	38.8	23.0	.....
L-Lysine	20	5	37.3	22.3	3.0
DL-Methionine	10	5	34.6	19.2	16.5
L-Glutamate	5	6	32.0	20.6	10.4

* Twenty per cent reduction needed to be significant.
† Values represent results from 90 animals fed Diet 802.

Materials and Methods

The 30-day cotton rat assay[2] was used in these studies. Littermates were fed the diets when 16–17 days old and given food and water ad libitum. The 802 cariogenic diet[3] containing 67 per cent sucrose was used, and the compounds tested were added at the expense of sucrose. Since the compounds were tested at levels no higher than 1.0 per cent, the reduction in sucrose was not sufficient to alter the cariogenicity of the diet. At the end of 30 days the animals were sacrificed and their teeth evaluated for caries.[3]

Amino acids, phenolic acids, polyphenols, antioxidants, and some compounds known to have antibacterial properties were tested.

All growth and caries-incidence data were analyzed statistically by analyses of variance and the *F*-test. Where significant differences were indicated, Duncan's multiple-range test was used to compare the means.[4]

Results and Discussion

The effect of the three amino acids (lysine, methionine, and glutamate) on the incidence of dental caries is shown in Table 1. No significant effect was observed on caries incidence or growth of the cotton rats. Statistical analyses reduction of 20 per cent was necessary to be significant in this experiment.

TABLE 2

EFFECT OF CERTAIN PHENOLIC ACIDS ON DENTAL CARIES

Compound	Conc. in Diet (g/kilo)	No. of Animals	Av. Gain (gm.)	Caries Incidence	Per Cent Reduction*
Cariogenic control†	.....	90	38.8	23	.....
Gallic acid	2	5	39.0	22	4.3
Syringic acid	1	15	37.6	14	39‡
Sinapic acid	1	5	38.3	20	13
Ferulic acid	2	6	37.3	23	0
Caffeic acid	5	5	38.9	24	0
P-Coumaric acid	2	5	39.6	23	0

* Twenty per cent reduction needed to be significant.
† Values represent results from 90 animals fed Diet 802.
‡ Significant at the 95 per cent level.

In Table 2 the effects of some phenolic

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acids are listed. A significant reduction in caries incidence was produced by syringic acid at a level of 0.1 per cent in the diet. None of the other acids caused a significant change in caries incidence at the levels tested. There were no marked effects on growth caused by any of the phenolic acids used in this study.

Of the polyphenols assayed, hesperidin, D-catechin, and hesperetin methyl chalcone caused a significant reduction in the incidence of caries as shown in Table 3. No effect was noted from hesperetin, morin, naringenin, and rutin at the levels tested. The glycoside, hesperidin, was much more effective than the aglycone, hesperetin, in reducing the incidence of caries; however, the chalcone of hesperetin caused a significant reduction. No adverse effects on growth were caused by any of the polyphenols which were assayed.

TABLE 3

EFFECT OF CERTAIN POLYPHENOLS ON DENTAL CARIES

Compound	Conc. in Diet (g/kilo)	No. of Animals	Av. Gain (gm.)	Caries Incidence	Per Cent Reduction*
Cariogenic control†	.....	90	38.8	23.0	.....
Hesperidin	3.0	15	37.4	13.0	43.5‡
Hesperetin	1.5	5	39.0	19.0	17.4
Morin	10.0	6	39.5	24.0	4.3
Narigenin	10.0	5	43.0	20.8	9.5
Rutin	23.0	4	38.1	23.0	0
D-Catechin	2.0	15	40.0	14.5	37‡
Hesperetin methyl chalcone	2.0	20	37.8	15.7	31.8‡

* Twenty per cent reduction needed to be significant.
† Values represent results from 90 animals fed Diet 802.
‡ Significant at the 95 per cent level.

As shown in Table 4, butylated hydroxyanisole and butylated hydroxytoluene caused a significant reduction in caries incidence. It was noted at the level fed that they also caused a significant reduction in growth. This may account for the effect on caries incidence. We have often observed that animals which exhibit poor growth usually have a lower incidence of caries when compared to animals growing well on the same diet.

Propylgallate was the only compound which was assayed that caused a significant increase in caries incidence. It did not have an effect on the growth rate. In contrast, nordihydroguaiaretic acid did not have an effect on caries incidence, but it did cause some reduction in growth at a level of 0.5 per cent in the diet.

The results in Table 5 show that no significant reduction in caries incidence was produced by 3-piperonylacrylic acid, lupulon, kojic acid, mandelic acid, or usnic acid. Usnic acid at levels of 0.1 and 0.2 per cent in the diet caused a significant reduction in growth. The other compounds listed in Table 5 were not toxic at the levels fed.

TABLE 4

EFFECT OF A FEW ANTIOXIDANTS ON DENTAL CARIES

Compound	Conc. in Diet (g/kilo)	No. of Animals	Av. Gain (gm.)	Caries Incidence	Per Cent Reduction*
Cariogenic control†	.....	90	38.8	23.0	.....
Nordihydroguaiaretic acid	5	10	30.6	20.4	11.3
Propylgallate	5	16	37.8	29.6	28.7
Butylated hydroxyanisole	5	16	27.2‡	13.4	41.7‡
Butylated hydroxytoluene	5	16	26.4‡	15.4	33‡

* Twenty per cent reduction needed to be significant.
† Values represent results from 90 animals fed Diet 802.
‡ Significant at the 95 per cent level.

A number of phenolic acids and polyphenols were present in extracts from oat hulls, and it was of interest to ascertain the effects of these compounds upon dental caries. From the results reported here it is evident that as a class these compounds are not highly active; however, certain compounds within these classes (syringic acid, hesperidin, D-catechin, and hesperetin methyl chalcone) do show significant activity.

Since considerable amounts of palmitic acid were present after hydrolysis of oat-hull extract, and with the above information it seemed likely that a combination of phenolic compounds and fatty acids might be effective anticariogenic agents. This was

tested with eugenol palmitate which was synthesized from eugenol and palmitoyl chloride. Eugenol alone did not reduce caries incidence, and palmitic acid was only slightly anticariogenic;[1] while eugenol palmitate produced a significant reduction in caries incidence when fed in the diet at a level of 0.3 per cent.

TABLE 5

EFFECT OF CERTAIN PHENOLIC ACIDS, POLYPHENOLS, AND EUGENOL PALMITATE ON THE INCIDENCE OF FISSURE CARIES

Compound	Conc. in Diet (g/kilo)	No. of Animals	Av. Gain (gm.)	Caries Incidence	Per Cent Reduction*
Cariogenic control†	.....	90	38.8	23.0	.....
3-Piperonyl-acrylic acid	5.0	5	35.2	22.8	0.9
Lupulon	2.0	7	39.0	21.9	4.8
Kojic acid	2.0	6	34.8	23.6	2.6
Mandelic acid	2.0	7	37.7	22.5	2.2
Usnic acid	0.05	6	33.1	21.8	5.2
	0.1	4	37.5	20.8	9.6
	0.5	5	31.6	19.2	16.5
	1.0	4	27.8‡	21.8	5.2
	2.0	4	16.2‡	23.0	0
Eugenol	1.0	6	35.8	24.6	6.0
Eugenol palmitate	3.0	6	37.1	17	26‡

* Twenty per cent reduction needed to be significant.
† Values represent results from 90 animals fed Diet 802.
‡ Significant at the 95 per cent level.

Other workers have reported activity from testing some of these compounds reported here with varying results. Jordan, Bowler, and Berger[5] using Sprague-Dawley rats, reported a 50 per cent reduction in incidence due to N-propylgallate, whereas we found this compound was associated with an increase in caries incidence. They reported that hesperidin did not inhibit caries, while we observed a reduction in incidence due to this supplement. They obtained results similar to those reported here for rutin and nordihydroguaiaretic acid. Lisanti and Eichel,[6] using hamsters, reported that nordihydroguaiaretic acid was effective in reducing caries in that species. They also reported that propylgallate inhibited caries. Gustafsson and Krasse[7] found that they could inhibit caries production in the hamster with naringenin. This compound did not have a significant effect on caries incidence in the cotton rat.

It has become increasingly evident that many factors play a role in influencing results from assays of this nature. We consider the following to be apropos: (1) the type of caries being studied; (2) species and strain of animal used; (3) length of the assay period; (4) method of administration; and (5) type of diet used.

Considerable caution must be exercised when extending results on caries inhibition beyond the experimental conditions under which they were obtained.

Summary

A number of organic compounds were tested for their effect on fissure caries in cotton rats. Syringic acid inhibited caries by approximately 40 per cent when incorporated in the diet at 0.1 per cent level. Other phenolic acids, including gallic acid, sinapic acid, ferulic acid, caffeic acid, and P-coumaric acid as well as the amino acids (lysine, methionine, and glutamate) did not inhibit caries. Hesperidin, D-catechin, and hesperetin methyl chalcone inhibited caries from 30 to 40 per cent when incorporated in the diet at 0.2 per cent level. Hesperetin, morin, naringenin, and rutin did not inhibit caries. Propylgallate at a level of 0.5 per cent in the diet caused an increase of about 30 per cent in the incidence of caries. Butylated hydroxyanisole and butylated hydroxytoluene inhibited caries 42 and 33 per cent, respectively, at a level in the diet of 0.5 per cent; however, this level also caused a reduction in growth rate. Nordihydroguaiaretic acid, 3-piperonylacrylic acid, lupulon, kojic acid, eugenol, mandelic acid, and usnic acid did not inhibit caries at the levels tested. Eugenol palmitate inhibited caries 26 per cent at a level of 0.5 per cent in the diet.

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From: Helvetica Chemica Acta. vol.XLI, fasc.II (1958)no.65-66 p.536-39.

65. Investigations of the fats of anti-oxidants in the animal body

I. On the behavior of the tert. butyl group

by Karl Bernhard and Hans Thommen

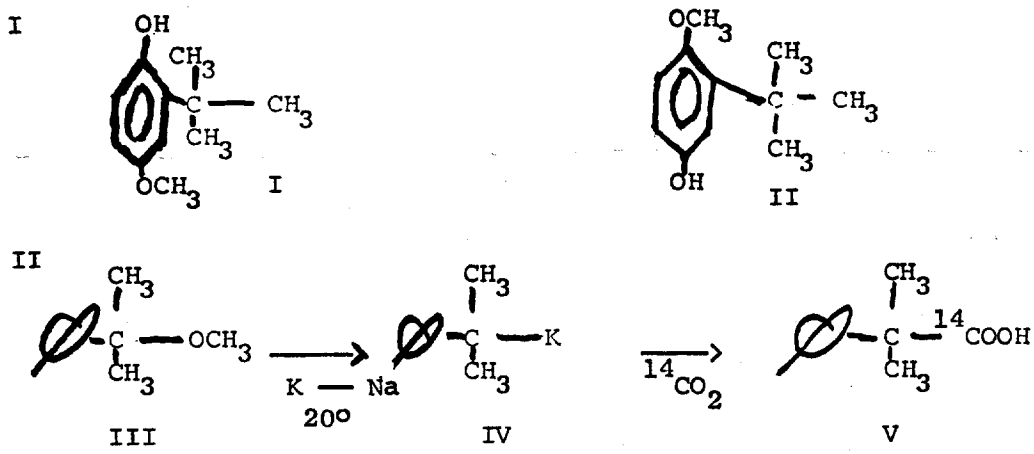
Dedicated to Prof. Dr. I. Abelin on his 75th birthday (1/25/1968)

The chemical substances added today to foods under various aspects present serious problems for nutrition researchers. The question regarding the fate of these substances in the human or animal organism is more than justified and can be answered in most cases. We have studied the behavior of the tert. butyl group found in some very common anti-oxidants, for example, t-butyl-hydroxy-anisol (BHA), which is used as an isomeric mixture of I and II, or of the somewhat less effective 2,6-di-t-butyl-4-methyl phenol or 2,6-di-t-butyl-phenol, and which is to a great extent responsible for the anti-oxidative action. (Reaction I)

Robinson and Williams (1) found after the administration of t-butyl benzene the glutoronide of 2,2-dimethyl-2-phenylethanol in rabbits at a rate of 90% of the applied dose, but no alpha-phenylisobutyric acid. The methyl-oxidation did not go beyond the state of the alcohol. Fed as such, it appeared almost quantitatively as a conjugate of glucoronic acid. Isopropyl benzene yielded both the glucoronides of the corresponding alcohol and of the acid, that is, of 2-methyl-phenyl-ethanol and of hydratropic acid (1).

The findings in the rabbit could deviate from the behavior of the tested compounds in organisms with a low pairing tendency, such as dog and rat. It is imaginable that alpha-phenyl isobutyric acid is also formed from tert. butyl benzene. It was therefore desirable to clarify the behavior of this possible intermediate product, particularly in quantitative respect.

Starting from ethyl-(2-phenyl-propyl-(2)-ether (III), we synthetized alpha-phenyl isobutyric acid (V) whose carboxyl group was marked 14-C over potassium-(2-phenylpropyl-(2): (Reaction II)



The specific activity was $1.082.10^6$ c/min.mg.

After dilution with inactive material, this compound was administered perorally to four male rats kept in metabolism cages. T he activities found in the urine and in the feces after a test period of 144 hours are compiled in table 1.

Table 1 Activity found in % of the applied activity

	animal			
	A	B	C	D
expiration carbon dioxide	0	0	0	0
urine	80.4	88.8	78.9	84.5
feces	5.4	2.3	5.5	5.6

In the animals C and D we also examined the gastrointestinal contents, the gastrointestinal duct, liver, kidneys and carcass for activity.

Table 2 Activities of the organs in % of the applied activity

	animal	
	C	D
gastrointestional duct	1.2	0.7
contents of gastrointestinal duct	0	0
liver	3.6	3.5
kidneys	0.9	4.2
kidney fat	0	0
carcass	0	0

Altogether 85.8, 91.1, 90.1 and 98.5% of the applied activity were found again.

The alpha-phenyl isobutyric acid could be isolated unchanged from the urine. Metabolites were not found. The total activity, as it could be measured after chromatographic separation, was due to the starting material.

Funds from the Hans Buss Stiftung were available for this study.

Experimental part

2-phenylpropanol-(2) The procedure indicated by Behal and Tiffeneau (2) was modified as follows: To a grignard solution of 48 g magnesium, 125 ml methyl iodide and 350 ml ether was added a solution of 120 ml acetophenone for 2 hours at -5 degC. The reaction mixture was left standing for 2 hours at room temperature and then poured over glacial hydrochloric acid. After the solvent was distilled off, a paleyellow oil remained. The fraction obtained at 84.5 - 85.5 degC consisted of pure carbinol. Yield. 85 g (63%).

(2-phenylpropyl-(2))-chloride Into a solution of 10 g phenyl-dimethyl-carbinol in 50 ml dry ether, to which 2-3 g anhydrous $MgSO_4$ had been added, we introduced under ice cooling dry HCl gas. The reaction product was washed with 2% $KHCO_3$ solution and water, dried over $MgSO_4$ and liberated of the solvent at room temperature by means of a water jet vacuum. Yield: 10 g (88%) crude chloride.

Methyl-(2-phenylpropyl-(2)-ether was prepared according to Ziegler and Schnell (3).

14-COOH-alpha-phenyl isobutyric acid In a three-necked flask we mixed an ether suspension of 0.1 g Na and 0.33 g K with 0.55 g purified methyl-(2-phenylpropyl-(2)-ether. After 4 hours the reaction vessel was evacuated on the high vacuum rake (4) and its contents frozen with liquid air. From 426 mg (0.2 mC) Ba 14-CO_3, 14-CO_2 was liberated with 15 ml conc. H_2SO_4, which was frozen out over the potassium-organic compound. On heating to room temperature, the reaction set in immediately. After acidification with 10 ml conc.

HCl the product was extracted with ether. The acid obtained from the ether was recrystallized from pentane. Melting point: 78.5 - 79.5 degC

Yield: 175 mg (50%)

Animal tests Phenyl isobutyric acid was administered to the rats as an aqueous solution of its Na-salt by means of a stomach sound. Further details can be seen from table 3.

Table 3

	animal			
	A	B	C	D
applied phenyl isobutyric acid,mg	113	113	83	107
applied activity, counts/min.	$1.30 \cdot 10^6$	$1.30 \cdot 10^6$	$1.10 \cdot 10^7$	$1.42 \cdot 10^7$
weight, g	240	245	390	320
duration of test, h	144	144	144	144

The active alkaline urine fractions of each animal were purified, concentrated, and these concentrates were liberated by means of preparative paper-chromatography (Schleicher-Schuell 2034b) of organic and coloring substances. The running time was according to the rising method in propanol-NH_3 (70:30) 8 hours. The active paper zone was cut out and eluted with propanol-NH_3. The eluates were extracted in the stirring extractor with petroleum ether in an acetic acid reaction. After recrystallization from pentane and preparation of the mother liquors, 60 mg (64%) of the applied phenyl isobutyric acid could be obtained in animal C and 80 mg (70.6%) inanimal D. They were identified by paper chromatography with a reference preparation, also by melting point- and mixed melting point determination and C, H-analysis.

The preparation of the organs was effected after careful homogenization and drying in known manner, and lipid-soluble and water-soluble portions were obtained.

Discussion of the results

After oral doses of carboxyl-marked phenyl isobutyric acid, the expiration

carbon dioxide remained inactive during the entire test period. Decarboxylation, which sets in in vitro on moderate heating of the acid in the vacuum, did not take place in the animal body. Metabolites could not be detected, nor conjugated products. The acid was rather eliminated practically completely in the urine.

After 144 hours the lipid extracts from the liver showed an activity of 3.6 and 3.5% resp. related to the applied quantities, which indicates a retention of the acid in the liver. The adipose tissue from the kidney regions, as well as the lipic extracts from the carcass were inactive. Aqueous extracts showed no activity either.

The appearance of phenyl isobutyric acid as an intermediate product of the decomposition of a tert. butyl group, as it is found in common antioxidants, would probably have to lead to complete elimination of the former in the urine, but by no means to decarboxylation or to extensive decomposition.

Summary

We prepared an alpha-phenyl isobutyric acid, marked 14-C in the carboxyl group, and administered it perorally to rats. It is not decomposed, rather it is eliminated again to a great extent in the urine, and only a small amount is retained in the liver. The finding is discussed with regard to the behavior of anti-oxidants with tert. butyl groups in the animal body.

Physical-Chemical Institute of the University of Basle

(Translated by Carl Demrick Associates,Inc/IE/t)

References

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[2]) *A. Béhal & M. Tiffeneau*, C. r. hebd. Séances Acad. Sci. 139, 140 (1904).
[3]) *K. Ziegler & B. Schnell*, Liebigs Ann. Chem. 437, 255 (1924).
[4]) *B. M. Tolbert*, J. Amer. chem. Soc. 69, 1530 (1947).

65. Untersuchungen über das Schicksal von Antioxydantien im Tierkörper.

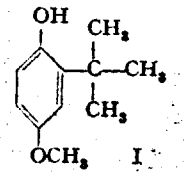
I. Zum Verhalten der tertiären Butylgruppe

von **Karl Bernhard** und **Hans Thommen.**

Herrn Prof. Dr. *I. Abelin* zum 75. Geburtstag gewidmet.

(25. I. 58.)

Unter den verschiedensten Aspekten heutzutage erfolgende Beimengungen chemischer Verbindungen zu Nahrungsmitteln stellen die Ernährungsforschung vor sehr ernsthafte Probleme. Die Frage nach dem Schicksal solcher Stoffe im menschlichen oder tierischen Organismus ist mehr als berechtigt und zumeist einer Beantwortung zugänglich. Wir haben im vorliegenden das Verhalten der tertiären Butylgruppe studiert, welche in einigen sehr gebräuchlichen Antioxydantien, etwa dem t-Butyl-hydroxy-anisol (BHA), das als Isomerengemisch von I und II verwendet wird, oder dem etwas weniger wirksamen 2,6-Di-t-butyl-4-methyl-phenol bzw. 2,6-Di-t-butyl-phenol, vorkommt und für die antioxydativen Eigenschaften weitgehend verantwortlich ist.



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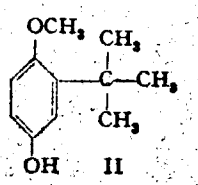


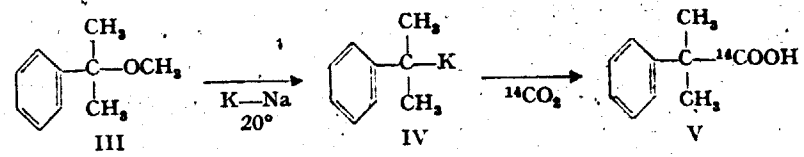
II

Robinson & Williams[1]) fanden nach Gaben von t-Butylbenzol am Kaninchen im Ausmasse von 90% der applizierten Dosis das Glucuronid des 2,2-Dimethyl-2-phenyläthanols, offenbar aber keine α-Phenylisobuttersäure. Die Methyloxydation führte nicht über die Stufe des Alkohols hinaus. Als solcher verfüttert, erschien dieser beinahe quantitativ als Glucuronsäure-Paarling. Isopropylbenzol gab sowohl die Glucuronide des entsprechenden Alkohols als der Säure, d. h. des 2-Methyl-phenyl-äthanols und der Hydratropasäure[1]).

Die Befunde an Kaninchen könnten vom Verhalten der geprüften Verbindungen in Organismen mit geringer Paarungstendenz, wie Hund und Ratte, abweichen. Es ist denkbar, dass aus dem tertiären Butylbenzol auch α-Phenylisobuttersäure entsteht. Daher war eine vor allem die quantitativen Verhältnisse berücksichtigende Abklärung des Verhaltens dieses möglichen Intermediärproduktes wünschenswert.

[1]) *D. Robinson & R. T. Williams*, Biochem. J. **59**, 153 (1955).

Wir synthetisierten ausgehend vom Methyl-[2-phenylpropyl-(2)]-äther (III) α-Phenylisobuttersäure (V), deren Carboxylgruppe über [2-Phenylpropyl-(2)]-kalium (IV) ^{14}C-signiert wurde:



Die spezifische Aktivität betrug $1{,}082 \cdot 10^6$ c/Min. mg.

Diese Verbindung verabreichten wir nach Verdünnung mit inaktivem Material peroral vier männlichen, in Stoffwechselkäfigen sich befindenden Ratten. Aus der Tabelle 1 sind die aufgefundenen Aktivitäten in Harn und Faeces nach einer Versuchsdauer von 144 Stunden ersichtlich.

Tabelle 1.

Aufgefundene Aktivität in % der applizierten Aktivität.

	Tier			
	A	B	C	D
Exspirationskohlensäure	0	0	0	0
Harn	80,4	88,8	78,9	84,5
Faeces	5,4	2,3	5,5	5,6

Bei den Tieren C und D haben wir auch den Magen-Darm-Inhalt, den Magen-Darm-Tractus, Leber, Nieren und Carcass auf Aktivität geprüft.

Tabelle 2.

Aktivitäten der Organe in % der applizierten Aktivität.

	Tier	
	C	D
Magen-Darmtractus	1,2	0,7
Inhalt des Magen-Darmtractus	0	0
Leber	3,6	3,5
Nieren	0,9	4,2
Nierenfett	0	0
Carcass	0	0

Insgesamt konnten 85,8, 91,1, 90,1 und 98,5% der applizierten Aktivität wieder aufgefunden werden.

Aus dem Harn liess sich die α-Phenylisobuttersäure unverändert isolieren. Metaboliten lagen nicht vor. Die gesamte Aktivität, wie sie nach chromatographischer Auftrennung gemessen werden konnte, entfiel auf das Ausgangsmaterial.

Für die vorliegende Arbeit standen Mittel der *Hans-Buss-Stiftung* zur Verfügung.

Experimentelles.

2-Phenylpropanol-(2). Die von *Béhal & Tiffeneau*[2]) angegebene Vorschrift wurde wie folgt abgeändert: Zu einer *Grignard-Lösung* von 48 g Magnesium, 125 ml Methyljodid und 350 ml Äther liess man bei – 5° und innerhalb 2 Std. eine Lösung von 120 ml Acetophenon fliessen. Das Reaktionsgemisch wurde 2 Std. bei Zimmertemperatur stehengelassen und dann auf Eis-Salzsäure gegossen. Nach dem Abdestillieren des Lösungsmittels blieb ein blassgelb gefärbtes Öl zurück. Die bei 84,5–85,5° gewonnene Fraktion bestand aus dem reinen Carbinol. Ausbeute: 85 g (63%).

[2-Phenylpropyl-(2)]-chlorid. Wir leiteten in eine Lösung von 10 g Phenyl-dimethylcarbinol in 50 ml trockenem Äther, die mit 2–3 g wasserfreiem $MgSO_4$ versetzt war, unter Eiskühlung trockenes HCl-Gas ein. Das Reaktionsprodukt wurde mit 2-proz. $KHCO_3$-Lösung und Wasser gewaschen, über $MgSO_4$ getrocknet und bei Zimmertemperatur mittels Wasserstrahlvakuum vom Lösungsmittel befreit. Ausbeute: 10 g (88%) Rohchlorid.

Methyl-[2-phenylpropyl-(2)]-äther wurde nach *Ziegler & Schnell*[3]) hergestellt.

^{14}COOH-α-Phenylisobuttersäure. In einem Dreihalskölbchen versetzten wir eine ätherische Suspension von 0,1 g Na und 0,33 g K mit 0,55 g gereinigtem Methyl-[2-phenylpropyl-(2)]-äther. Nach 4 Std. wurde das Reaktionsgefäss am Hochvakuumrechen[4]) evakuiert und sein Inhalt mit flüssiger Luft ausgefroren. Aus 426 mg (0,2 mC) $Ba^{14}CO_3$ setzten wir mit 15 ml konz. H_2SO_4 $^{14}CO_2$ frei, welches über der kalium-organischen Verbindung ausgefroren wurde. Beim Erwärmen auf Zimmertemperatur erfolgte sofortige Reaktion. Nach 12stündigem Stehenlassen wurde die überschüssige Legierung mit 10 ml Äthanol zersetzt. Nach Ansäuern mit 10 ml konz. HCl erfolgte Extraktion mit Äther. Die aus dem Äther gewonnene Säure kristallisierten wir aus Pentan um. Smp.: 78,5–79,5°. Ausbeute: 175 mg (50%).

Tierversuche. Die Phenylisobuttersäure wurde als wässerige Lösung ihres Na-Salzes mit Hilfe einer Magensonde an die Ratten verabreicht. Alle näheren Angaben sind aus der Tabelle 3 ersichtlich.

Tabelle 3.

	Tier			
	A	B	C	D
Appl. Phenylisobuttersäure, mg	113	113	83	107
Appl. Aktivität, counts/Min.	$1{,}30 \cdot 10^6$	$1{,}30 \cdot 10^6$	$1{,}10 \cdot 10^7$	$1{,}42 \cdot 10^7$
Gewicht, g	240	245	390	320
Dauer des Versuches, Std.	144	144	144	144

Die aktiven alkalischen Harnfraktionen eines jeden Tieres haben wir vereinigt, eingeengt und diese Konzentrate mittels präparativer Papierchromatographie (*Schleicher-Schüll* 2034b) von anorganischen und färbenden Bestandteilen gereinigt. Die Laufzeit betrug nach der aufsteigenden Methode in Propanol-NH_3 (70:30) 8 Std. Die aktive Papierzone wurde ausgeschnitten und mit Propanol-NH_3 (70:30) eluiert. Die Eluate extrahierten wir im Rührextraktor mit Petroläther bei essigsaurer Reaktion. Nach Abdampfen des Lösungsmittels erhielt man ein Rohkristallisat von 65 bzw. 85 mg. Nach Umkristallisieren aus Pentan und Aufarbeiten der Mutterlaugen konnte beim Tier C 60 mg (64%), beim Tier D 80 mg (70,6%) der verabreichten Phenylisobuttersäure erhalten werden. Ihre Identifikation erfolgte papierchromatographisch mit einem Vergleichspräparat, ferner durch Schmelzpunkt- und Mischschmelzpunkt-Bestimmung und C,H-Analyse.

Die Aufarbeitung der Organe geschah nach sorgfältiger Homogenisierung und Trocknung in üblicher Weise unter Gewinnung lipidlöslicher und wasserlöslicher Anteile.

[2]) *A. Béhal & M. Tiffeneau*, C. r. hebd. Séances Acad. Sci. **139**, 140 (1904).
[3]) *K. Ziegler & B. Schnell*, Liebigs Ann. Chem. **437**, 255 (1924).
[4]) *B. M. Tolbert*, J. Amer. chem. Soc. **69**, 1530 (1947).

Diskussion der Ergebnisse.

Nach oralen Gaben von Carboxyl-signierter Phenylisobuttersäure blieb während der ganzen Versuchsdauer die Exspirationskohlensäure inaktiv. Eine Decarboxylierung, die *in vitro* beim mässigen Erwärmen der Säure im Vakuum eintritt, fand im Tierkörper nicht statt. Metaboliten konnten nicht nachgewiesen werden, desgleichen auch keine Paarungsprodukte. Die Säure wurde vielmehr praktisch vollständig im Harne wieder ausgeschieden.

Nach 144 Stunden zeigten die Lipidextrakte aus der Leber eine Aktivität von 3,6 bzw. 3,5%, bezogen auf die applizierten Mengen, was auf eine gewisse Retention der Säure in der Leber hindeutet. Das Fettgewebe aus den Nierenbezirken, ebenso die Lipidextrakte aus dem Carcass, waren inaktiv. Auch in den wässerigen Auszügen war keinerlei Aktivität nachweisbar.

Das Auftreten von Phenylisobuttersäure als Intermediärprodukt des Abbaues einer tertiären Butylgruppe, wie sie in gebräuchlichen Antioxydantien vorkommt, müsste voraussichtlich zu einer völligen Ausscheidung ersterer im Harne, keinesfalls aber zu einer Decarboxylierung oder zu einem tiefgehenden Abbau führen.

Zusammenfassung.

Wir haben eine in der Carboxylgruppe ^{14}C-signierte α-Phenylisobuttersäure hergestellt und an Ratten peroral verabreicht. Sie wird nicht abgebaut, vielmehr grösstenteils im Harn wieder ausgeschieden und nur geringfügig in der Leber zurückgehalten. Der Befund wird im Hinblick auf das Verhalten von Antioxydantien mit tertiärer Butylgruppe im Tierkörper diskutiert.

Physiologisch-Chemisches Institut der Universität Basel.

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66. Über N-Amino-α-pyridone

von K. Hoegerle[1]).

(25. I. 58.)

In einer vorläufigen Mitteilung[2]) haben wir kürzlich über den Umsatz von α-Pyridon mit Chloramin berichtet. Es wurde bei dieser Reaktion eine Verbindung der Summenformel $C_5H_6ON_2$ isoliert, der auf Grund von vergleichenden pK-Messungen und von IR.-Spektren die Struktur eines 1-Aminopyridons-(2) (I) zugeordnet werden konnte.

Diese neuartige Methode der N-Aminierung von α-Oxo-N-heterocyclen wurde in der Folge auf methylsubstituierte α-Pyridone sowie auf Verbindungen der Chinolin- und Thiazolreihe übertragen. Im folgenden beschreiben wir die Herstellung der neuen Verbindungen und ihre Charakterisierung durch ihr chemisches Verhalten sowie durch die IR.- und UV.-Spektren.

[1]) Auszug aus Diss. *K. Hoegerle*, Basel 1957.

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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

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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. Dugan, Marx, Weir & Kraybill (1954) 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 & Bianchine (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 G. 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 & Chettleburgh, 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 60 mg. (total recovery 64%). 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 *iso*butylene 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 90·0% 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 (1954) as modified by El Masry, Smith & Williams (1956). 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) Gibbs 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 9·24). (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. 208–209° (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 0·2 M-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 (1946). 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, 72·5; 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 (90:10:1); 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, 278, 284 ~ †	8030, 2200
3:5-Di-*tert.*-butyl-4-hydroxybenzoic acid	0·45–0·5	0·8	0·9	M	YO	YG	214, 260	26560, 13145
3:5-Di-*tert.*-butyl-4-hydroxybenzaldehyde	0·65	0·8	—	pM	None	pYG	227·5, 290	18720, 16615
3:5-Di-*tert.*-butyl-4-hydroxybenzyl alcohol	0·4	0·8	0·9–1·0	M	Y	YG	249, 254·5, 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	261	13440
β-(3-*tert.*-Butyl-2-hydroxy-5-methylphenyl)-$\beta\beta$-dimethylethanol	0·6	0·8–0·85	0·9–1·0	pY	None	pB	283	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.), $[\alpha]_D^{17\cdot5}$ −45·0° in $CHCl_3$ (c, 1·0); light-absorption max., 208 and 279 mμ (ε 10 490 and 2900 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, 25·0; mol.wt., 541. $C_{28}H_{40}O_{11}$ 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 (104 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 (1958). 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. 160° (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.), $[\alpha]_D^{20}$ −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., 86·4%), 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., 93·9%). 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, 0·667 ± 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 12·90 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	—	22·4	24·4	33·7	—	—	9·0	—
1	3·64	4·1	19·9	22·0	35·4	18·4	(17·0)	9·9	—
4	1·82	9·6*	—	—	—	—	—	—	—
4	3·65	—	—	—	33·2	14·9	(18·3)	—	—
5	3·63	—	21·2	—	36·1	16·0	(20·1)	7·6	—
5	3·64	8·0	—	—	—	—	—	10·3	1·8
5	3·64	—	30·9	30·7	36·6	14·5	(22·1)	6·0	—
	Mean	8·4	24·1	27·2	35·9	16·0	(19·4)	8·0	1·8

* 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.)	
	2·25	4·70
Non-conjugated phenols	5·2	2·0
Free phenols (non-conjugated phenol + ester glucuronide)	90·5	—
Total phenols	96·2	—
Total glucuronides	84·0	87·5
Ester-type glucuronide	83·5	—
Glycine conjugate	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)-$\beta\beta$-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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