Biochemical Pharmacology, Vol. 26, pp. 2145–2149. Pergamon Press, 1977. Printed in Great Britain.

EFFECTS OF MONOCYCLIC COMPOUNDS ON BIOMEMBRANES*

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(*Received* 4 *October* 1976; *accepted* 15 *March* 1977)

Abstract—Monocyclic compounds with an aliphatic chain containing at least 2 carbon atoms, interact with biomembranes producing solubilization of proteins. Plasma membrane seems to be more sensitive than mitochondrial–lysosomal membrane to the damaging effects of these compounds. Studies on the structure–activity relationship have shown that benzene derivatives were more active than other compounds having either a etherocyclic or a saturated hexacarbon ring. Among tert.-butylbenzene derivatives, 2,tert.-butyl,4 methoxyphenol (BHA) and 3,5,di-tert.-butyl, 4, hydroxytoluene (BHT) exerted the most remarkable effects. It is suggested that the electronic density on the aromatic nucleus plays an important role on the interaction of monocyclic compounds with membrane constituents.

Since the reaction of oxidative deterioration of polyunsaturated lipids, is supposed to be the cause *in vivo* of some pathological modifications which occur in aging processes, in atherosclerosis, in ethanol liver injury, etc. [1], the administration of antioxidants-free radical scavengers has been encouraged as a rational tool in preventing or reducing damage in animals induced by poisons which stimulate peroxidation processes [2]. However, attempts to verify *in vitro*, on subcellular components, the protection exerted by antioxidants against membrane damage which accompanies lipid peroxidation, have been so far disappointing [3].

The effects of the antioxidant food additives BHA and BHT on living organisms have been extensively studied. When used at concentrations lower than 10^{-3} M, BHT is a potent inactivator of lipid-containing mammalian and bacterial viruses [4], BHA inhibits growth of some Gram-positive bacteria† and BHT inhibits growth of monolayer cultures of mammalian cells [5]. Our finding that BHA and BHT labilize rat liver lysosomal membrane [3] would indicate that membranes may be their main site of action. These effects have been suggested to be due to the lipid solubility of these compounds [6]. However, our observation [3], that BHA and not BHT, which has a higher lipid solubility [6], is able to release glutamic acid dehydrogenase activity from rat liver mitochondria, prompted us to undertake more detailed studies on the interactions between these compounds or their structural analogues and biomembranes.

MATERIALS AND METHODS

BHA‡, BHT, tert.-butylacetate, thiobarbituric acid, tert.-butylcyclohexane, butylcyclopentane, 4-tert.-butylpyridine, *N*-butylimidazole, *n*-propylbenzene ("purum" reagents), tert.-butylalcohol, tert.-butylamine, ethylbenzene ("purissimum" reagents and octylbenzene ("practicum" reagent) were purchased from Fluka A.G. Buchs S.G.; 2,2,4-trimethylpentane (analytical grade) and methoxybenzene (95%) from BDH Chemical Ltd; *n*-butylbenzene, iso-butylbenzene, sec-butylbenzene and tert.-butylbenzene (analytical grade) from Eastman Kodak Co.; pyridine, *o*-methoxyphenol, phenol, benzene, cyclohexane, ethanol and dimethylsulfoxide (Uvasol® reagents) from E. Merk, Darmstadt; iso-butylphenylpropionic acid from the Boots Co., Nottingham. All the other compounds used were analytical grade reagents.

Experiments with rat liver mitochondria–lysosomes suspensions. Sprague–Dawley rats of both sexes, 200–250 g, were used throughout. They were given food§ and water *ad lib.* Mitochondria–lysosomes (M–L) suspensions were prepared from livers as previously described [3]. Aliquots (corresponding to 3–100 mg of protein suspension) were incubated at 37° for 60 min in air atmosphere with continuous shaking in a medium containing 250 mM sucrose, 126 mM dimethylsulphoxide (DMSO) plus studied compounds at the specified concentrations, adjusted to pH 6.8. Following incubation, a 2 ml aliquot of the mixture was deproteinized with 5% TCA and assayed for peroxide, while the remainder was centrifuged at 20,000 *g* for 20 min and the clear supernatant thus obtained was used for protein and enzyme activity assays. Protein and enzyme activities were also measured in the M–L suspensions added with 25 ml of 0.1% Triton-X-100 in order to obtain total contents.

* Presented in part before the Società Italiana di Biologia Sperimentale at Florence, October 7th, 1975.

† Unpublished observation.

‡ The compound used was free from detectable amounts of nitrogen and chlorates, according to qualitative analyses performed in our laboratory.

§ According to the rat-food manufacturing company (Soc. S. Morini, S. Polo D'Enza, Italy) 1 kg of rat pellets contained: vitamin A, 15,000 i.u.; vitamin D_3, 1,500 i.u.; vitamin K_3, 2 mg; vitamin E, 30 mg; vitamin B_1, 3 mg; vitamin B_2, 5 mg; vitamin C, 40 mg; *d*-pantothenic acid, 15 mg; vitamin B_6, 3 mg; vitamin B_{12}, 0.03 mg; folic acid, 1 mg; vitamin PP, 30 mg; choline chloride, 400 mg; manganese, 60 mg; iron, 150 mg; copper, 5 mg; zinc, 30 mg; iodine 1 mg; cobalt, 0.2 mg; BHT, 10 mg.

Protein content was determined colorimetrically by the method of Lowry *et al.* [7], using bovine serum albumin as standard. The samples were previously extracted with chloroform to avoid any interference of the tested compounds on the assay. Protein assay in samples containing Triton-X-100 was performed by a modification of the Lowry method [8].

Acid phosphatase (EC 3.1.3.2) activity was measured as described by Linhardt and Walter [9], using *p*-nitrophenylphosphate as substrate.

L-Glutamic acid dehydrogenase (EC 1.4.1.2) (GLDH) activity was measured by the method of Schmidt [10]. Lipid peroxides were determined by the thiobarbituric acid (TBA) test [11]. The incubation was performed at 80° instead of 100° in order to avoid any interference produced by sucrose [12]. Values were expressed as TBA index (extinction at 535 nm.mg^{-1} of protein).

Experiments with erythrocytes. Erythrocytes were isolated from blood of different mammals and washed 3 times with 154 mM NaCl. Each time the cells were pelleted by centrifugation at 1,400 g for 20 min. Red cells were freely sacrificed in all wash steps to ensure complete removal of buffy coat. The supernatant was clear and colorless after the final wash. The entire procedure was carried out at room temperature. Incubation of 3–5 × 10^8 cells (corresponding to 15–25 mg of protein) was performed at 37° in air atmosphere for different periods of time in a medium containing 154 mM NaCl, 126 mM DMSO (medium A) and the tested compounds at the specified concentrations; in some experiments, the medium (medium B) contained in addition 15 mM Na-phosphate buffer, pH 7.0 and 5 mM glucose. After incubation the cell suspension was centrifuged at 1,400 *g* for 10 min. Readings at 540 nm of the supernatant before and after the cell suspension had been lysed with 0.1% Triton-X-100, gave the amount of hemoglobin released by tested compounds expressed as percentage of the total content. Methemoglobin, formed during the incubation, was assayed as described by Stolman [13] and expressed as percentage of total hemoglobin according to Vandenbilt *et al.* [14].

RESULTS

Effects on rat liver mitochondria–lysosomes suspensions. The amounts of protein, acid phosphatase and glutamate dehydrogenase activity released from M–L preparations incubated with 10 μmoles of BHA or BHT are reported in Table 1. BHA released protein in amounts which were inversely related to the amount of protein present in the incubation medium.

In the presence of a total amount of 100 mg of protein BHA was ineffective, producing a protein leakage of 2.5 per cent as obtained under control conditions. In the presence of 26.8 mg of protein the effect produced by BHT was less extensive than that provoked by BHA. A partial explanation of this differential effect is given by a study on the release of enzyme activities. BHA and BHT were equally active on releasing acid phosphatase activity. However, while BHA also released a significant amount of GLDH activity, the release of this enzyme activity was not affected by BHT.

In order to gain some insight on this different behaviour the effects exerted by compounds structurally related to BHA and BHT upon the release of proteins, acid phosphatase and GLDH activity from M–L suspensions were studied. All compounds were tested at 1 mM concentration. Results are summarized in Table 2. Compounds from group 3, but not toluene, were all able to produce the release of protein. Benzene derivatives were more potent than other compounds possessing either an etherocyclic ring or a saturated hexacarbon ring. This is shown by the fact that while tert.-butylcyclohexane produced only a 250 per cent increase of protein and a 700 per cent increase of acid phosphatase release and had no effect upon the release of GLDH activity, tert.-butylbenzene produced a much higher effect on the release of protein and acid phosphatase activity (400 and 1100 per cent respectively) and furthermore it also produced an extensive increase (500 per cent) of GLDH release. The observation that toluene was inactive while ethylbenzene, *n*-butylbenzene and octylbenzene produced an increase in the release of proteins of 215, 335 and 225 per cent respectively, would suggest that the length of the aliphatic chain also has a role in the observed effects. All studied compounds had no direct effect on the tested enzyme activities. These findings seem to indicate that the interaction of the aromatic nucleus with membrane constituents plays an important role on the observed effects Further support to this indication comes from experiments where the

Table 1. Effects of BHA and BHT on the release into the medium of proteins, GLDH and acid phosphatase activities from rat liver M–L suspensions

Added compound	Protein (mg)		GLDH (i.u.)		Acid phosphatase (i.u.)	
	Total	Released	Total	Released	Total	Released
BHA	9.9 ± 2.0	4.9 ± 1.4 (44.4)	2.8 ± 0.3	1.3 ± 0.4 (46.0)	3.0 ± 0.0	2.8 ± 0.2 (93.0)
	26.8 ± 0.8	5.2 ± 0.6 (19.3)				
	43.0	6.2 (14.4)				
	100.0	2.5 (2.5)				
BHT	26.8 ± 0.8	2.3 ± 0.3 (8.5)	2.8 ± 0.3	0.4 ± 0.0 (14.3)	3.0 ± 0.0	2.8 ± 0.2 (93.0)

Figures represent mean values ± S.E.M. derived from 15 experiments. Figures in brackets represent the released amounts expressed as percentage of total content. Under control conditions (0.126 M DMSO alone) the amounts of GLDH and Acid phosphatase activity released accounted for 14 and 6 per cent respectively.

Table 2. Effects of monocyclic compounds on rat liver M–L suspensions: release of proteins, acid phosphatase and glutamate dehydrogenase and lipid peroxides content

Added compound	Protein (%)	Acid phosphatase (%)	GLDH (%)	Lipid peroxides (%)
None	100	100	100	100
1				
Tert.-butylalcohol	95	93	90	100
Tert.-butylamine	102	85	90	100
Tert.-butylacetate	102	80	—	98
Trimethylpentane	95	80	78	95
2				
Pyridine	100	—	—	75
Benzene	95	100	117	150
Cyclohexane	100	100	105	90
3				
Tert.-butylpiridine	230	—	—	75
N-Butylimidazole	235	—	—	53
n-Butylcyclopentane	180	—	—	58
Toluene	105	—	—	95
Ethylbenzene	215	—	—	35
Propylbenzene	185	—	—	60
n-Butylbenzene	335	1565	135	78
Sec.-butylbenzene	503	1430	175	66
Iso-butylbenzene	300	1600	—	60
Tert.-butylbenzene	370	1140	525	38
Tert.-butylcyclohexane	250	694	97	80
Octylbenzene	200	—	—	35
4				
Phenol	115	85	154	70
Anisole	103	80	95	78
o-Methoxyphenol	120	85	128	40
Iso-butyl-*p*-phenylpropionic acid	100	—	—	—

M–L suspensions (26.8 ± 0.8 mg of proteins) were incubated in the presence of different compounds at 1 mM concentration. Figures represent mean values from 3 to 10 experiments, expressed as percentage of values found in control conditions (0.126 M DMSO alone).

release of proteins induced by BHA was studied in the presence of solvents having different dipole moments. Results are summarized in Table 3. Under control conditions, about 5 per cent of the total protein content was found in the supernatant, DMSO, ethanol and dioxane all seemed to protect the particles from this spontaneous release. However, this protective effect was statistically significant only in the case of DMSO ($P < 0.05$). The release of protein produced by 1 mm BHA was significantly higher when DMSO was the solvent used as compared to ethanol or dioxane. DMSO has been recently recognized as a membrane stabilizer [16]. In agreement with this finding DMSO reduced the spontaneous leakage of protein (see Table 3) and was able to prevent the spontaneous swelling of rat liver mitochondria suspended in 0.25 M sucrose (data not reported here). The stabilizing effect of DMSO has been interpreted as evidence for decreased membrane fluidity [16] consequently, if any the effect of DMSO should be of reducing the perturbing action of BHA. Protein leakage promoted by BHA is higher in the presence of the solvent having a higher dipole moment in spite of its stabilizing property. This finding may suggest

Table 3. Release of proteins from rat liver M–L suspensions* induced by BHA in the presence of different solvents

Solvent	Protein released (mg)			Dipole moment$_D$	Ref.
	− BHA	+			
None	1.7 ± 0.2				
DMSO	1.2 ± 0.2†	5.1 ± 0.2		3.870	§
Ethanol	1.3 ± 0.4	4.5 ± 0.4†		1.441	[15]
Dioxane	1.3 ± 0.2	4.3 ± 0.2‡		0.450	§

* 31.72 ± 0.15 mg of proteins incubated as described under Methods in the presence of 1 mM BHA and different solvents 126 mM. Figures represent mean values ± S.E.M. derived from 5 to 15 experiments. The significance of the difference between treatments was tested by the analysis of variance.

† Significantly different from the appropriate control at $P < 0.05$.

‡ Significantly different from the appropriate control at $P < 0.01$.

§ Spectroquality solvents; Spectra, physical properties, specifications and typical uses, MC/B Manufacturing Chemists, Norwood (1971).

Table 4. Effects of monocyclic compounds on erythrocytes: release of hemoglobin

Added compound	Erythrocytes		
	man	guinea pig	rat
None	4	16	12
1			
BHA	12	70	35
BHT	97	95	100
2			
Benzene	15	—	—
Pyridine	4	6	—
3			
Tert.-butylpyridine	54	44	89
N-Butylimidazole	8	—	—
Toluene	5	33	35
n-Butylcyclopentane	23	93	89
Ethylbenzene	75	90	83
Propylbenzene	65	99	94
n-Butylbenzene	94	—	—
Sec.-butylbenzene	96	—	—
Iso-butylbenzene	97	—	—
Tert.-butylbenzene	88	90	86
Tert.-butylcyclohexane	8	—	—
Octylbenzene	9	46	56
4			
Phenol	2	—	—
Anisole	9	—	—
o-Methoxyphenol	1	—	—
Iso-butyl-*p*-phenyl-propionic acid			
pH 4.3	4	20	—
pH 6.4	5	18	—

Erythrocytes were incubated in medium A as described under Methods. All compounds were added at 1 mM concentration. The hemoglobin released is expressed as percentage of the total hemoglobin content. Figures represent mean values derived from 6 to 10 experiments.

that DMSO can influence the action of BHA by enhancing the electron density on the ring through an induction mechanism.

Effects on erythrocytes. The effects exerted by monocyclic compounds on blood red cells from man, guinea pig and rat are summarized in Table 4. All compounds were tested at 1 mM concentration. BHT caused an almost complete lysis of cells from all species, while BHA caused release of 12, 70 and 35 per cent of the total hemoglobin from human, guinea pig and rat erythrocytes respectively. Among group 3 compounds ethylbenzene, propylbenzene and butylbenzene isomers produced an almost complete hemolysis. It is interesting to observe that the effect of tert.-butylcyclohexane on human erythrocytes was negligible. Group 4 compounds, including iso-butyl-*p*-phenylpropionic acid were practically devoid of hemolytic activity. Incubation of BHA with human erythrocytes, while produced hemolysis to a small extent, induced methemoglobin formation. After 3 hr incubation of human red cells with BHA 98 per cent of the total hemoglobin was oxidized to methemoglobin. When glucose was present in the incubation medium (medium B) a longer time of incubation, i.e. 6 hr, was needed to obtain the same extent of methemoglobin formation.

DISCUSSION

As shown in this report, monocyclic compounds like BHA and BHT, possessing an aliphatic chain with at least two carbon atoms, in spite of their antioxidant properties (see Table 2), exert a primary perturbing action on biomembranes which causes structural modifications resulting in a lysis of erythrocytes, oxidation of human hemoglobin and release of proteins from rat liver mitochondria and lysosomes.

In separate experiments the ratios (μmoles of the compound.mg^{-1} of protein of the preparation used) at which BHT exerted half maximal effects on red cell lysis and on protein release from M–L suspensions were found to be 0.015 and 1 respectively. This suggests that perturbation of cell membrane is the effect produced by the lowest concentration.

In a study on the ability, shared by some phenol derivatives like BHA and BHT, to inhibit RNA synthesis on primary cultures of monkey kidney cells, it has been emphasized that this activity correlates to the lipid solubility of the tested compounds [5]. Our study on the structure–activity relationship has shown that benzene derivatives were more active than other compounds having either an etherocyclic or a saturated hexacarbon ring. According to these results it seems that the higher the electron density on the ring, the greater are the effects of monocyclic compounds on biomembranes. Thus, these effects cannot be reasonably restricted to the solvent property of these molecules.

We suggest that most of the action sustained by monocyclic compounds depends upon their interaction with hydrophobic and electrophilic regions of the membrane. It is worth noting, in this respect. that iso-butyl-*p*-phenylpropionic acid, although having lipophilic properties, did not seem to affect membranes.

Partial support to this hypothesis comes from a recent study carried out by Eletr *et al.* [17]. In this study BHT has been compared to adamantane as a perturbing agent of axial symmetry of packed chains on phospholipid vesicles. The solubilization of a molecule such as adamantane, a saturated hydrocarbon of quasi-spherical shape, in lipid hydrophobic regions causes disruption of the symmetry of the solid state. The same happens with BHT; however, the resulting disruption reflected by the data shown on the Arrhenius plot is not as simple as that shown in the case of adamantane, presumably due to the dual nature of the hydrophobic and amphiphilic character of BHT.

It is probable that the hydroxyl which is not so hindered in BHA as it is in BHT molecule may account also for the peculiar effects exerted by BHA on human erythrocytes. Prior to inquiring about the biochemical events that might support the hemoglobin oxidation, however, one must exclude any direct effect of BHA on hemoglobin. It is noticeable that *p*-methoxy-phenol and 3,5-di-tert.-butylsalicilic acid chelate Fe^{3+} bound to non-heme proteins [18]. If this is the case with BHA, the oxidation of human hemoglobin could depend on the fact that methemoglobin, naturally formed in the cell, once bound to BHA, might be no longer accessible to specific reducing systems. The low rate at which hemoglobin was

oxidised by BHA in our experiments suggests this mechanism.

Acknowledgements—This research has been supported by the grant CT 75.00628.04 from the Consiglio Nazionale delle Ricerche-Roma. We wish to thank Prof. S. Ruggieri for his helpful discussion and Mr F. Ciuffi, Mrs Marta Galardi and Mrs Marigrazia D'Asta for their technical assistance.

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