Biochemistry (Moscow), Vol. 67, No. 11, 2002, pp. 1271-1275. Translated from Biokhimiya, Vol. 67, No. 11, 2002, pp. 1539-1544.

Ionol (BHT) Produces Superoxide Anion

E. G. Smirnova[1], Yu. I. Lyubimov[2], T. G. Malinina[2], E. Yu. Lyubimova[2], N. I. Alexandrushkina[3], B. F. Vanyushin[3], G. M. Kolesova[3], and L. S. Yaguzhinsky[3]*

[1]Institute of Agricultural Biotechnology, Russian Academy of Agricultural Sciences, Timiryazevskaya ul. 42, Moscow, 127550 Russia
[2]Scientific-Production Complex "Biotechindustry", 2-i Spaso-Nalivkovsky Pereulok 6, Moscow, 119991 Russia
[3]Belozersky Institute of Physico-Chemical Biology, Lomonosov Moscow State University, Moscow, 119899 Russia; fax: (095) 939-3181; E-mail: yag@genebee.msu.su

Received November 8, 2001
Revision received December 17, 2001

Abstract—In aqueous medium etiolated wheat seedlings release superoxide anion (O_2^-). Interaction of a synthetic antioxidant, butylated hydroxytoluene (BHT, ionol), with oxygen in the aqueous medium is accompanied by O_2^- formation. This suggests that under certain conditions BHT behaves as a prooxidant. A natural antioxidant, superoxide dismutase (SOD), and also a wound healing preparation, emulsified denatured placenta (EDP), do not exhibit the prooxidant properties. In contrast to BHT, they reduce O_2^- production by the etiolated wheat seedling system.

Key words: BHT, antioxidant, prooxidant, wheat seedlings, reactive oxygen species, superoxide

Superoxide anion (O_2^-) is generally considered as a reactive particle which may damage (at high concentrations) cellular structures. However, it becomes evident that endogenously generated O_2^- plays a certain positive role in cell functioning. Low concentrations of O_2^- regulate key metabolic processes. Under certain conditions O_2^- stimulates cell division (mitosis) [1-3], in other cases it triggers programmed cell death (apoptosis) [4, 5].

The existence of a natural antioxidant system including α-tocopherol, superoxide dismutase, catalase, glutathione reductase, etc. controls the content of reactive oxygen species (ROS) in the cell [6]. Since O_2^- is involved in the regulation of mitosis and apoptosis this antioxidant system not simply inhibits ROS generation to the lowest level as thought earlier, but maintains a certain concentration of ROS. In the light of new notions on the biological role of ROS it is important to study in more detail the properties of synthetic and natural antioxidants.

Using three day old wheat seedlings we investigated earlier the effect of butylated hydroxytoluene (BHT) on early stages of morphogenesis in plants [7]. In these experiments BHT was added to the seedling medium on the second day of the seedling growth and plants contacted with this substance in darkness at 26°C for 48 h. Under these conditions BHT sharply decreased superoxide generation by seedlings. However, this was accompanied by impairments in morphogenesis, changes in growth rate and shape of seedlings [7]. This is consistent with the notion on superoxide anion as a factor regulating mitosis and apoptosis. This also suggests that alteration of normal (cellular) level of superoxide impairs normal plant development.

In the present study we found that under certain conditions BHT can behave as a prooxidant, effectively reducing molecular oxygen by a one electron mechanism with superoxide anion formation. We also compared BHT effect on generation of superoxide anion by wheat seedlings [7] with effects of superoxide dismutase and another natural antioxidant, emulsified denatured placenta (EDP).

MATERIALS AND METHODS

Seedlings of Moskovskaya-39 winter wheat (*Triticum aestivum* L.) were used in the experiments [7].

The rate of superoxide anion generation was determined as described previously [7, 8]. Two three-day-old etiolated seedlings were washed with water and transferred into 4 ml of assay medium, containing 10 mM

Abbreviations: BHT) butylated hydroxytoluene (2,6-di-*tret*-butyl-4-methyl-phenol, ionol); NBT) nitro blue tetrazolium; EDP) emulsified denatured placenta; ROS) reactive oxygen species; SOD) superoxide dismutase.

* To whom correspondence should be addressed.

0006-2979/02/6711-1271$27.00 ©2002 MAIK "Nauka/Interperiodica"

phosphate buffer, pH 7.8, 10 μM EDTA (disodium salt), 0.1% Triton X-100 (Fluka, Switzerland), and superoxide anion scavenger, 0.6 mM nitro blue tetrazolium (NBT, Sigma, USA). Superoxide anion formed in the surface seedling tissues and released under our experimental conditions into the aqueous medium was registered by reduction of NBT to formazan as described in [8, 9]. Seedlings were incubated in the medium in darkness at 26°C for 1 h.

Specificity of O_2^- registration was controlled in parallel samples, containing bovine erythrocyte superoxide dismutase (SOD, Sigma) (50 U/ml), which almost totally inhibited NBT reduction (see Fig. 3). In these experiments the amount of superoxide generated was determined as the difference between reduced NBT (formazan) absorbance at 530 nm in samples with and without SOD (Fig. 1) using molar coefficient of formazan absorbance of 15,000 $M^{-1} \cdot cm^{-1}$ [9]. All measurements were carried out using a Hitachi 557 spectrophotometer (Japan).

BHT (final concentration 0.23 mM) was added into the medium after 24 h of seed soaking. (Before transfer of seedlings into the medium they were washed with water.) In some experiments seedlings were cultivated without BHT, which was added (in final concentration of 0.5 mM) into the medium together with seedlings. (NBT was initially present in the assay medium.)

For analysis of possible superoxide generation by BHT itself we used seedling-free assay medium, containing 10 mM phosphate buffer, pH 7.8, 10 μM EDTA (disodium salt), 0.1% Triton X-100, and various concentrations of BHT (from 40 to 200 μM) and NBT (from 0.3 to 1.2 mM).

The preparation of emulsified denatured placenta (EDP) obtained from Scientific-Production Complex "Biotechindustry" [10] has complex composition and contains CoQ and copper salts.

RESULTS AND DISCUSSION

It was previously shown that BHT added to the seedling medium sharply decreased superoxide generation [7]. In the present study we found abnormal effect of this antioxidant. BHT added to three day old seedlings stimulated the rate of superoxide generation instead of inhibiting it (Figs. 1 and 2b). This phenomenon was qualitatively reproduced in various seasons and in many experiments, although its magnitude varied (Table 1).

In contrast to BHT, natural antioxidants SOD and EDP always inhibited superoxide generation of seedlings (Figs. 3 and 4) and never stimulated superoxide generation. At high concentrations EDP totally inhibited superoxide generation (Fig. 4); SOD (≥50 U/ml) inhibited this reaction by 95% (Fig. 3).

Since the magnitude of the stimulating effect of BHT on superoxide generation was rather variable (Table 1), we repeated experiments of our previous study [7] in which BHT was added to the medium of plant seedling on the second day of growth. Figure 2a shows that in accordance with the previously published results [7], BHT

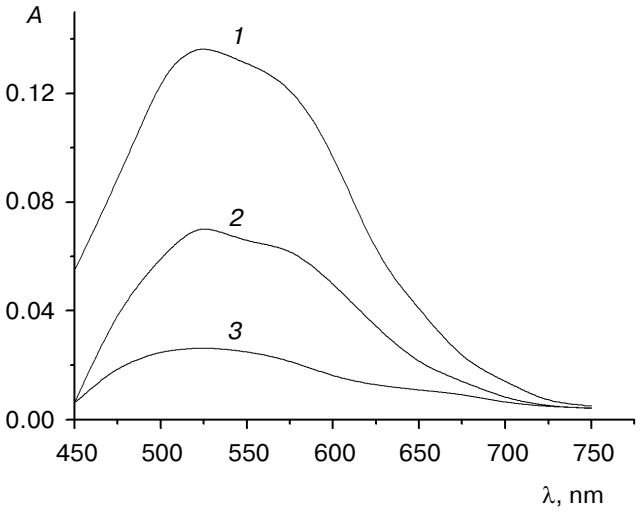


Fig. 1. Effect of BHT and SOD on the rate of O_2^- generation by wheat seedlings. *1-3*) Absorbance spectra of monoformazan, the product of NBT reduction by superoxide anion: *1*) seedlings + NBT (0.6 mM) + BHT (0.5 mM); *2*) seedlings + NBT (0.6 mM) (control); *3*) seedlings + NBT (0.6 mM) + SOD (25 U/ml).

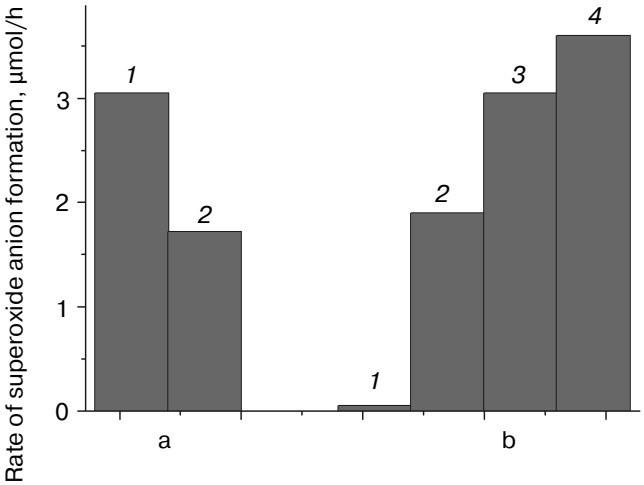


Fig. 2. Effect of BHT on the rate of O_2^- formation at various conditions (the result of a typical experiment). a) BHT in the seedling medium: *1*) control seedlings (without BHT); *2*) seedlings + BHT (0.23 mM); b) BHT in the assay medium: *1*) assay medium without seedlings and BHT; *2*) assay medium without seedlings + BHT (0.5 mM); *3*) assay medium + seedlings without BHT; *4*) assay medium + seedlings + BHT (0.5 mM).

Table 1. Effect of 0.5 mM ionol on the rate of superoxide anion (formazan) accumulation in the assay system aqueous medium–seedling

Experiment number	Superoxide generation, μmol/h		
	– ionol	+ ionol	difference
1	1.55	2.60	1.05
2	1.60	2.55	0.95
3	1.60	2.65	1.05
4	2.90	3.50	0.60
5	3.05	3.70	0.65
6	3.20	3.55	0.35

acted as an effective antioxidant reducing superoxide anion generation.

Understanding of the dual effect of BHT requires answers to the following questions: 1) How does this antioxidant accelerate superoxide anion formation? 2) Why does it exert an opposite effect under certain conditions?

To answer the first question we studied O_2^- formation in the standard system containing BHT and NBT but lacking seedlings (Fig. 5). In these experiments the rate of O_2^- formation was also measured as the difference of formazan content formed in two parallel samples (with and without added SOD). In the absence of seedlings O_2^- was formed due to direct interaction of BHT with molecular oxygen.

According to these experiments the amount of superoxide formed per 1 h was comparable to the amount of superoxide generated per 1 h by seedlings in the absence of BHT (see Fig. 2b). Quantitative experiments revealed almost linear accumulation of superoxide during the initial period of incubation (Fig. 5a, curves *2-4*). During this period there was a linear relationship between BHT concentration (from 40 to 200 μM) and the rate of superoxide generation (Fig. 5b). Thus, the kinetics of the reaction between BHT and oxygen did formally correspond to a bimolecular process rather than a chain mechanism. Control experiments also revealed that increase in NBT concentration from 0.6 to 1.2 mM insignificantly influenced the rate of superoxide generation in the medium (Table 2). This suggests that NBT does not react with oxygen effectively and therefore it cannot be considered as an additional source for superoxide generation in the model system BHT–NBT–oxygen. This conclusion is also supported by kinetic data (Fig. 5a, curve *1*). In the presence of SOD (25-50 U/ml) the formation of colored products (formazans) was not detected. This also suggests that NBT reduction occurs only via O_2^- formation.

Thus, the model experiments provided convincing evidence that O_2^- can be formed in the reaction of oxygen reduction by BHT (ionol) in the aqueous medium.

In the previous study [7] we demonstrated that the presence of BHT in the seedling cultivation medium inhibited O_2^- formation. In these experiments BHT was not added to the O_2^- assay medium. Clearly, the major quantity of antioxidant penetrated into the plant tissue had to be accumulated inside the seedling. Under these conditions the antioxidant effect may be attributed to BHT (ROH) interaction with reactive radicals, particu-

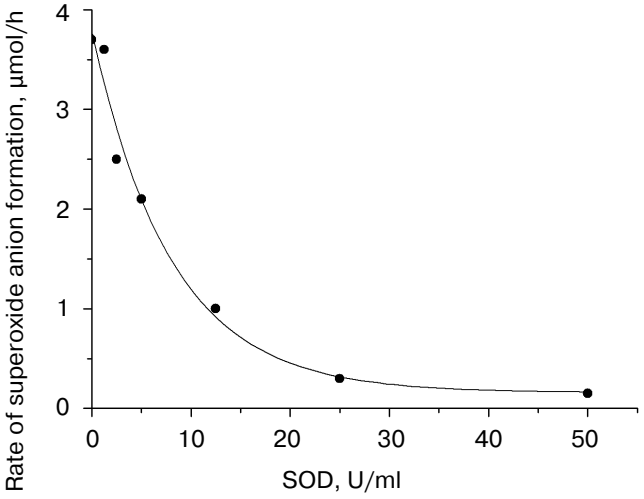


Fig. 3. Dependence of the rate of O_2^- generation by wheat seedlings on the amount of SOD in the assay medium.

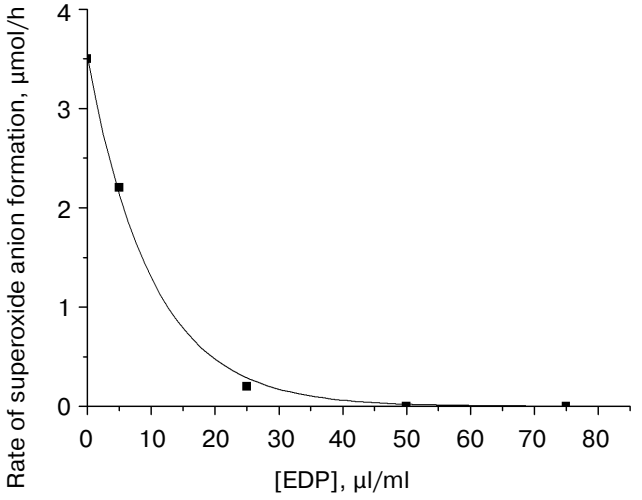


Fig. 4. Dependence of the rate of O_2^- generation by wheat seedlings on the amount of EDP in the assay medium.

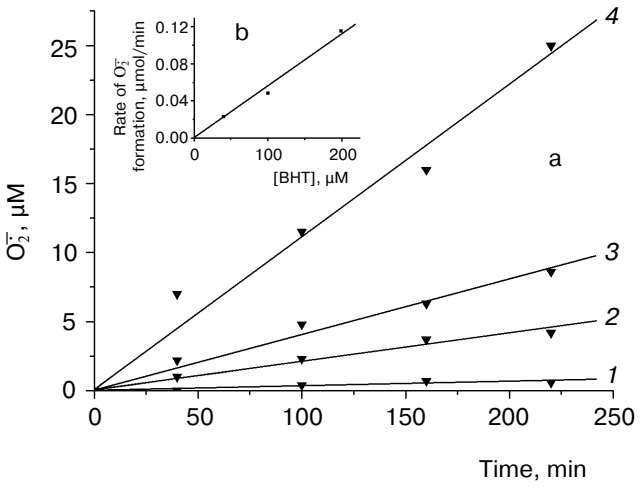


Fig. 5. Kinetics of O_2^- formation during BHT oxidation in the aqueous phase of the model system. The incubation medium contained 10 mM phosphate buffer, pH 7.8, 10 μM EDTA (disodium salt), 0.1% Triton X-100, 0.6 mM NBT. a) Rate of O_2^- formation at various BHT concentrations: *1*) without BHT (control); *2*) 0.04 mM BHT; *3*) 0.1 mM BHT; *4*) 0.2 mM BHT. b) The dependence of O_2^- generation on BHT concentration.

larly, with O_2^- ($^{\cdot}O_2H$) followed by formation of relatively stable phenoxyl radical (RO$^{\cdot}$) (reaction (1)):

$$ROH + {}^{\cdot}O_2H \rightarrow [RO^{\cdot} + H_2O_2] \rightarrow RO^{\cdot} + H_2O + 1/2\,O_2. \quad (1)$$

In the present study we changed experimental conditions and cultivated seedlings without BHT and added this substance only to the O_2^- assay medium together with seedlings and NBT. In this case the excess of BHT was outside the seedling in the aqueous phase saturated with oxygen. Such experimental conditions promote additional superoxide formation to be registered (Fig. 1). Results of model experiments (Fig. 5) suggest that superoxide anion formation may occur via reactions (2) and (3) independently of superoxide generation by the plant (see below). Addition of natural O_2^- scavengers, SOD (Figs. 1 and 3) or EDP (Fig. 4), instead of BHT significantly decreased the rate of O_2^- formation.

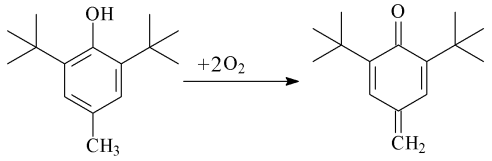
Table 2. Concentrations of O_2^- (formazan) registered in the reaction of 0.5 mM ionol with oxygen at different time intervals in the presence of two concentrations of nitro blue tetrazolium (NBT)

[NBT], mM	$[O_2^-]$, μM			
	40 min	100 min	160 min	220 min
0.6	7.0	11.5	16.0	25.0
1.2	5.5	11.7	16.0	24.6

Earlier the possibility of superoxide anion formation was demonstrated during interaction of BHT with oxygen in the presence of a catalyst (vanadium) [11]. In the present study we found that this process (O_2^- formation) may occur without catalyst (via reaction (2)). In this case BHT acting as prooxidant interacts with molecular oxygen rather than with O_2^- radical. As in the case of reaction (1), this reaction (2) yields phenoxyl radical and superoxide [12]:

$$ROH + O_2 \rightarrow RO^{\cdot} + {}^{\cdot}O_2H. \quad (2)$$

Deeper oxidation of BHT may result in formation of BHT-quinone methide (reaction (3)) and other products of BHT oxidation which were described earlier [13, 14]:



(3)

It should be noted that the products of BHT oxidation can also generate superoxide anion [13].

In the system employed in our experiments both types of reactions, reaction (1) and reactions (2) and (3), are quite possible. The effective run of reaction (1) would be observed in the presence of increased concentrations of $^{\cdot}O_2H$; according to the law of action mass the contribution of reactions (2) and (3) should increase during the increase in molecular oxygen concentration.

Discussing dual effects of BHT on the rate of O_2^- formation it is reasonable to assume that this is due to different distribution of BHT in the aqueous phase–seedling system. When BHT is added to the assay medium just before measurement the major part of BHT remains in the aqueous phase. This increases probability of BHT interaction with oxygen. The possibility of spontaneous reactions between these substances was demonstrated in our experiments presented in this paper (Fig. 5).

The BHT-induced increase of ROS in animal tissues has already been demonstrated [15]. However, the mechanism of this phenomenon was qualitatively different; it was related to inhibition of cellular antioxidant systems.

In contrast to BHT, natural antioxidants (EDP and SOD) did not exhibit prooxidant properties under our experimental conditions. Similarity in behavior of EDP and SOD may be plausibly attributed to the presence of cooper salts in EDP preparations. (Copper salts are known to possess some superoxide dismutase activity [16].)

Effective production of superoxide anion by BHT (ionol) in aerobic condition requires certain revision of chemical and pharmacological properties of synthetic antioxidants. This class of important biologically active compounds influences cellular level of superoxide anion, which is involved in regulation of key processes in the cell, such as apoptosis and mitosis. So, synthetic antioxidants require exhaustive study of their properties especially in the case of their use as antioxidants as food additives [12, 14, 17] or medical drugs.

Now it is firmly recognized that BHT employed in some countries as a food preservative has potent carcinogenic effect [17-21]; BHT can induce lung cancer and hepatic tumors [22]. BHT can also cause cell polyploidy in the liver [23] and stimulation of epidermal cell proliferation [24]. BHT administration to phenobarbital-pretreated animals induces hepatic cell death [25]. These biological properties of BHT may be well explained by the characteristic feature of this preparation, described in the present paper, i.e., superoxide anion production in the reaction with oxygen.

This work was supported by the Russian Foundation for Basic Research (grants 99-04-48090, 00-15-97920, 01-04-48647, 01-04-48566, and 00-04-48257).

REFERENCES

1. Brar, S. S., Kennedy, T. P., Whorton, A. R., Murphy, T. M., Chitano, P., and Hoidal, J. R. (1999) *J. Biol. Chem.*, **274**, 20017-20026.
2. Yeh, L. H., Park, Y. J., Hansalia, R. J., Ahmed, I. S., Deshpande, S. S., Goldshmidt-Clermont, P. J., Irani, K., and Alevriadou, B. R. (1999) *Am. J. Physiol.*, **276**, 838-847.
3. Lee, S. L., Wang, W. W., Lanzillo, J., Gillis, C. N., and Fanburg, B. L. (1998) *Biochem. Pharmacol.*, **56**, 527-533.
4. Jabs, T. (1999) *Biochem. Pharmacol.*, **57**, 231-245.
5. Bauer, G., Dormann, S., Engelmann, L., Schulz, A., and Saran, M. (2000) in *Handbook of Experimental Pharmacology* (Cameron, R. G., and Feuer, G., eds.) Vol. 142, Springer Verlag, N. Y., pp. 275-318.
6. Salgo, M. G., and Pryor, W. A. (1996) *Arch. Biochem. Biophys.*, **333**, 482-488.
7. Shorning, B. Yu., Smirnova, E. G., Yaguzhinsky, L. S., and Vanyushin, B. F. (2000) *Biochemistry (Moscow)*, **65**, 1357-1361.
8. May, M. J., Hammond-Kosack, K. E., and Jones, D. G. (1996) *Plant Physiol.*, **110**, 1367-1379.
9. Auclair, C., and Voisin, E. (1987) in *CRC Handbook of Methods for Oxygen Radical Research* (Greenwald, R. A., ed.) CRC Press, Inc., Boca Raton, Florida, pp. 123-132.
10. Lyubimov, Yu. I., Malinina, T. G., and Lyubimova, E. Yu. (1995) Russian Patent No. 2033797 (Registered in State List of Inventions 30.04.95).
11. Gekhman, A. E., Stolarov, I. P., Moiseeva, N. I., Rubaijlo, V. L., Vargaftik, M. N., and Moiseev, I. I. (1998) *Inorg. Chim. Acta*, **275/276**, 453-461.
12. Kagan, V. E., Serbinova, E. A., and Packer, L. (1990) *Arch. Biochem. Biophys.*, **280**, 33-39.
13. Nagai, F., Ushiyama, K., and Kano, I. (1993) *Arch. Toxicol.*, **67**, 552-557.
14. Oikawa, S., Nishino, K., Oikawa, S., Inoue, S., Vizutani, T., and Kawanishi, S. (1998) *Biochem. Pharmacol.*, **56**, 361-370.
15. Vartaniyan, L. S., and Gurevich, S. M. (1999) *Vopr. Med. Khim.*, **45**, 314-320.
16. Ramadan, A. M., and El-Naggar, M. M. (1996) *J. Inorg. Biochem.*, **63**, 143-153.
17. Omaye, S. T., Reddy, K. A., and Cross, C. E. (1977) *J. Toxicol. Environ. Health*, **3**, 829-836.
18. Thompson, J. A., Carlson, T. J., Sun, Y., Dwyer-Nield, L. D., and Malkinson, A. M. (2001) *Toxicology*, **160**, 197-205.
19. Thompson, J. A., Bolton, J. L., and Malkinson, A. M. (1991) *Exp. Lung Res.*, **17**, 439-453.
20. Lindenschmidt, R. C., Margaretten, N., Griesemer, R. A., and Witschi, H. P. (1986) *Carcinogenesis*, **7**, 1581-1586.
21. Witschi, H. R., and Morse, C. C. (1983) *J. Natl. Cancer Inst.*, **71**, 859-866.
22. Briggs, D., Lok, E., Nera, E. A., Karpinski, K., and Clayson, D. B. (1989) *Cancer Lett.*, **46**, 31-36.
23. Haesen, S., Derijcke, T., Deleener, A., Castelain, P., Alexandre, H., Preat, V., and Kirsch-Volders, M. (1988) *Carcinogenesis*, **9**, 1755-1761.
24. Kuzuya, M., Naito, M., Funaki, C., Hayashi, T., Yamada, K., Asai, K., and Kuzuya, F. (1991) *Artery*, **18**, 115-124.
25. Powell, C. J., and Connolly, A. K. (1991) *Toxicol. Appl. Pharmacol.*, **108**, 67-77.