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BUTYLHYDROXYANISOL (BHA), BUTYLHYDROXYTOLUENE (BHT) AND ETHOXYQUIN (EMQ) TESTED FOR MUTAGENICITY

By

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JONER, P. E.: *Butylhydroxyanisol (BHA), butylhydroxytoluene (BHT) and ethoxyquin (EMQ) tested for mutagenicity.* Acta vet. scand. 1977, *18*, 187—193. — The antioxydants butylhydroxyanisol (BHA), butylhydroxytoluene (BHT) and ethoxyquin (EMQ) were tested for mutagenicity in the so-called "Ames test". The test was performed with and without the addition of microsomal mixed function oxidase system from rat liver. The compounds were found not to be mutagenic in this test. At high concentration of BHA and EMQ toxic effects on the growth of the bacteria were observed.

mutagenicity; Ames test; feed additives; antioxydants.

Food and feed additives are substances of highly diverse structure and function. It is therefore difficult to establish a uniform set of experimental procedures to test the safety in detail for all of them. Most countries have developed, however, a general pattern of experiments that they require by legislation to be carried out before a substance is allowed to be used as a food or feed additive.

Relevant experiments are acute toxicity studies, biochemical studies, short term and long term toxicity studies and studies concerning reproduction, embryotoxicity, teratogenicity and mutagenicity/carcinogenicity. Among these the acute, the short term and the long term toxicity studies are in common use. From such studies are calculated the Acceptable Daily Intake (ADI) values of the different additives. Recent research has shown that the usual toxicological studies ought to be supplemented and

more emphasis be put on studies concerning mutagenic, carcinogenic and allergic effects of established as well as potential additives.

Recently Bruce Ames and coworkers have introduced a test that seems to offer promising potentialities as to carcinogenicity/mutagenicity (*Ames et al.* 1973, 1975, *McCann et al.* 1975). They have worked out a system measuring mutagenicity in certain bacteria based on the hypothesis that all carcinogens are also mutagens. The advantage of studying mutagenesis in a bacterial system is of course the time factor and the ease with which bacterial mutations can be registered.

A set of test strains of Salmonella typhimurium that carry certain special properties have been selected for this purpose. The bacteria are revertable mutants in the histidine operon that are deleted in the uvr repair system (the enzymatic system of bacteria that repairs damage on the cells' DNA caused by i.e. uv radiation) and have a weakened cell wall polysaccharide. Under the influence of a mutagen they revert from histidin requirement to prototrophy and can thus be registered as visible colonies on a minimal medium. Certain carcinogenic compounds are not detected in this system because they are not carcinogenic before they are activated by the microsomal mixed function oxidases of the mammalian liver. Ames and coworkers have solved this problem by including extracts of mammalian liver in their bacterial system. They use a liver extract that consists of a supernatant from homogenized liver of animals that have been induced to form increased microsomal mixed function oxidases by treatment with PBC, phenobarbital or methylcholantrene.

In our laboratory we are presently engaged in the investigation of some food and feed additives with special emphasis on their potential mutagenic activity as measured with Ames' test. As a part of this work we have studied the antioxydants that are the subject of this report.

MATERIALS AND METHODS

Chemicals

0- and p tert. butylhydroxyanisol (practical grade) was obtained from Fluka AG Chemische Fabrik, Switzerland, butylhydroxytoluene Tenox BHT (food grade) from Eastman Chemical Products Inc. and ethoxyquin (pure) from Koch-Light Laboratories Ltd. PCB-aroclor 1254 from Monsanto Company, was kindly donated by dr. Steinar Øvrebø, the Department of Biochemistry, University of Bergen, Norway.

Bacterial strains

TA 1535, TA 1537, TA 1538, TA 98 and TA 100 of Salmonella typhimurium were kindly donated by Bruce Ames, University of California. The bacteria were cultivated over night in nutrient broth when received, quickly frozen in a dry ice/ethanol mixture after addition of DMSO (0.8 ml bacterial culture and 0.07 ml DMSO) and kept at —80°C. When used, fresh inoculations directly from the frozen stock cultures were done into nutrient broth weekly. The cultures were kept at 4°C between experiments. Before use the strains were checked as to histidin requirement, deletion of repair function, increased permeability and for TA 98 and TA 100 presence of R factor.

Other components of the system

Minimal medium plates. The salt basis contained 0.2 g $MgSO_4 \cdot 7H_2O$, 2.0 g citric acid, 10 g K_2HPO_4 and 3.5 g $NaNH_4HPO_4 \cdot 4H_2O$ per l. To this was added glucose to 2 % and agar to 1.5 % final concentration.

Top agar. To 0.6 % agar and 0.5 % NaCl was added 5 ml of a sterile solution 0.5 mM in histidin and biotin. Histidin is added to give the bacteria the possibility to grow. Mutagens can only exert their effect on growing cells.

S9-mix. Per ml this mixture contained:

0.5 ml of a Na/K-phosphate buffer, 0.2 M, pH 7.4
40 µl of a 0.1 M solution of NADP
20 µl of a solution that is 0.4 M in $MgCl_2$ and 1.65 M in KCl
5 µl of a solution of 1 M glucose-6-phosphate
0.1 ml S9
water up to 1.0 ml

The S9 was a supernatant from homogenized liver from rats that had received 5×1.0 ml PCB (2 mg/ml). The protein content was 30 mg/ml.

Performance of the test

The substance to be tested for mutagenicity was dissolved in DMSO and diluted with the same solvent to the final concentration to be used in the experiment. The order of addition of components of the system to the top agar was as follows:

1. Bacteria (0.1 ml)
2. Substance to be tested (usually in 0.1 ml)
3. S9-mix (0.5 ml)

The last addition and the pouring of the soft agar onto the plates were carried out as quickly as possible to prevent inactivation of the liver enzymes.

Positive control experiments

To assure that the system was working we carried out control experiments with benz(a)pyrene as mutagen for TA 1537, TA 1538,

Table 1. Mutagenicity experiments with ethoxyquin (a), butylhydroxytoluene (b) and butylhydroxyanisol (c). The experiments were carried out by adding the compounds at the concentrations indicated to soft agar containing bacteria and fortified liver extract (S9-mix) and pouring the soft agar mixture onto minimal agar plates. After incubation at 37°C for 2 days the revertant colonies were counted. The columns headed by —EMQ, —BHT and —BHA give the number of spontaneous revertants; tox.: means that in these plates we registered false positives as a result of the high concentration toxicity of the compounds.

a. Ethoxyquin (EMQ)

Strain	10 μg per plate				100 μg per plate				1000 μg per plate			
	+ S9		— S9		+ S9		— S9		+ S9		— S9	
	—EMQ	+EMQ	—EMQ	+EMQ	—EMQ	+EMQ	—EMQ	+EMQ	—EMQ	+EMQ	—EMQ	+EMQ
TA 1535	21	20—25	12	10—7	17	22—23	12	11—11	17	tox.	16	0—0
TA 1537	9	4—4	6	9—1	3	1—3	6	6—6	10	3	5	0-tox.
TA 1538	14	17	21	11—13	16	23—11	21	15—11	22	0	13	0—0
TA 98	25	29—30	16	14—24	18	22—13	16	16—9	62	67	29	0—4
TA 100	111	77—82	124	137—125	98	91—77	124	100	146	tox.	189	0—0

b. Butylhydroxytoluene (BHT)

Strain	10 μg per plate				100 μg per plate				1000 μg per plate			
	+ S9		— S9		+ S9		— S9		+ S9		— S9	
	—BHT	+BHT	—BHT	+BHT	—BHT	+BHT	—BHT	+BHT	—BHT	+BHT	—BHT	+BHT
TA 1535	14	19	19	15—20	16	13—12	19	17—19	12	15—18	7	8—6
TA 1537	2	5—6	3	4—8	13	5—6	3	4—6	5	5—12	5	3—9
TA 1538	25	21—20	20	24—16	25	16—14	21	23—15	15	13—16	22	15—25
TA 98	14	14—16	23	18—20	24	29—31	23	26—18	26	24—18	16	8—15
TA 100	80	84—82	116	97—79	115	128	110	88—79	118	106—110	17	120—121

c. Butylhydroxyanisol (BHA)

Strain	10 μg per plate				100 μg per plate				1000 μg per plate			
	+ S9		— S9		+ S9		— S9		+ S9		— S9	
	—BHA	+BHA	—BHA	+BHA	—BHA	+BHA	—BHA	+BHA	—BHA	+BHA	—BHA	+BHA
TA 1535	20	21—35	25	29—20	42	53	20	34—29	13	0—0	16	0—3
TA 1537	14	13	15	15—12	5	5	15	12—7	3	0—3	5	0—0
TA 1538	36	32—33	18	22—13	26	18	18	15—25	15	0—3	13	0—0
TA 98	22	21—24	19	27	43	34	19	22—24	23	0—0	29	0—0
TA 100	80	97—74	122	134—97	136	62	122	140—153	80	0—0	189	0—0

TA 98 and TA 100 and with NaN_3 as mutagen for TA 1535. The positive results were in accordance with comparable results in other laboratories (*McCann et al.* 1975).

RESULTS

Butylhydroxytoluene, butylhydroxyanisol and ethoxyquin were tested in concentrations of 10—1000 μg per plate. In Table 1 is shown the results of the experiments. As seen from the table none of the antioxydants seemed to be mutagenic in the Ames system at the concentrations investigated. The compounds were tested with and without the addition of the S 9 fraction. At high concentrations of butylhydroxyanisol and ethoxyquin toxic effects were observed. To determine more closely at what concentration the toxicity appeared, ethoxyquin was studied further in the concentration range 100 to 1000 μg per plate with TA 1535 and TA 100 (Table 2). At concentrations of 600—800 μg per plate the toxicity of the compound gave results that at first were mistaken as positive as to mutagenic effect. What really happened, however, was that the killing of most of the bacteria made more of the small amount of histidin added available to the few survivors and these grew to appear as visible colonies on the plates. The fenomenon has been described earlier by *Ames* (1973). As seen from Table 2 TA 100 seems to be more sensitive to the toxic effect of the compounds.

Table 2. Mutagenicity experiments with increasing concentration of ethoxyquin. The experiments were carried out as described in the legend to Table 1. The column headed by 0 gives the number of spontaneous revertants.

	Amount of ethoxyquin added per plate in μg					
Strain	0	100	2000	400	600	800
TA 1535	7	8—25	7—10	6—10	6—10	tox.
TA 100	146	112—113	160—157	146—166	tox.	tox.

DISCUSSION

The investigation of the antioxidants butylhydroxytoluene, butylhydroxyanisol and ethoxyquin using Ames's test shows that these compounds are not mutagenic as such, and further that rat liver mixed function oxidases do not convert them to mutagenic derivatives. The close correlation between mutagenic and carcinogenic effect leads us to the conclusion that these compounds

probably are not carcinogenic either. There has been a thorough discussion in the literature recently as to the reliability of different short time tests for carcinogenicity and to what extent the tests for mutagenic effects can be used to predict carcinogenicity in man (*Stoltz et al.* 1974, *Rubin & Ames* 1976, *Sivak* 1976, *Matter* 1976). In a recent review article on this problem *Bridges* (1976) claims that Ames's test is the short time test that has the highest percentage of correct carcinogenicity predictions (90 % of known human carcinogens are mutagenic in Ames' test) where as in vitro cell transformation came close to 83 %, morphological changes following subcutaneous implantation about 70 % and tetrazolium reduction in mouse skin predicted 62 % of known carcinogens correctly.

We do not consider the results, showing that the three antioxidants are not mutagenic, as conclusive evidence for these compounds to be completely harmless. The results must be judged in connection with other studies. It seems, however, that one is safer when using substances that turn out to be negative in Ames' test in food or feed than when using substances that have not been tested in this system. Consequently we recommend that Ames' test or equivalent short time bacterial tests for mutagenicity should be included in the routine investigations of any compound used as a food or feed additive.

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SAMMENDRAG

Butylhydroksytoluen, butylhydroksyanisol og ethoxyquin testet for mutagenitet.

Antioksydantene butylhydroksytoluen (BHT), butylhydroksyanisol (BHA) og ethoxyquin (EMQ) er undersøkt med hensyn til mutagenisitet i den såkalte "Ames test" med negativt resultat. Undersøkelsen har vært utført både med og uten tilsetning av mikrosomalt blandet oksydase system fra rottelever fra dyr indusert med PCB. Ved høye konsentrasjoner av butylhydroksyanisol og ethoxyquin kan man observere toksisk virkning på bakterienes vekst.

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