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Individual and Combined Effect of Commonly Some Consumed Food Additives

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Abstract
Different types of food additives such as preservatives and coloring agents are widely used in food processing and have different technological functions. This investigation aimed to study the cytotoxicity effect of some food additives which are commonly used. Most of the studies investigated the individual effect of food preservatives and coloring agents although its combinedly consumed. The study followed, the cytotoxicity effect of analyzed food additives performed by MTT test using normal lung cells (wi-38 cells). The chosen additives were potassium benzoate (E 212) which is existed in candy products as a preservative, in addition to sodium nitrite (E 251) that are used in cured meat products for inhibiting *Clostridium botulinum* growth and maintaining the bright red color of the cured meat were considered. Allura red (E 129) which existed in food additive legislation, and was also studied. The examined additives had significant cytotoxicity effect on normal lung cells ($P<0.05$) and its viability were also affected by preservatives when individually used or together with others owing to combination of studied items was higher than the individual one.
Keywords : Food Additives, Preservatives, Coloring agents, Cytotoxicity Effect, MTT test.

1. Introduction

According to the Codex Alimentarius Standard (2023), food additive means “any substance not normally consumed as a food by itself and not normally used as a typical ingredient of the food, whether or not it has nutritive value , the intentionally addition of which to food for a technological (including organoleptic) purpose in the manufacture, processing ,preparation, treatment,, packing, packaging ,transport or holding of such food stuff or may be reasonably expected to result (directly or indirectly) , in it or its by-products becoming a component of or otherwise affecting the characteristics of such foods . The term does not include
contaminants or substances added to food for maintaining or improving nutritional qualities.

Regarding the increase of food additives in the last two decades, studies were increasingly documented endocrine disruption and other adverse health effects. In many cases, exposure to these chemicals is disproportionate among low-income populations (Leonardo *et al.,* 2018). The assessment of food additives is worldwide supported by control system of the Acceptable Daily Intake (ADI), which developed by Jecfa (2017). The importance of technological additives in food production is evident (Tfouni and Toledo 2002), it is necessary to be aware of the toxicological risks that caused by large quantities and frequent consumption of these substances (Sifa , 2007).

The use of food additives is justified if they are complying with the regulations and does not cause any hazard effect or risk to consumers in the prescribed concentration. Because of the specificity of the child's metabolic system, there is a greater risk that of the food additive will manifest (Marijana *et al.*, 2022).

Over 10,000 different food additives have been approved for use in the food field. The most used food additives in children’s food are food colors, preservatives, sweeteners, and flavor enhancers (Laura *et al.* 2019).

Food preservation is one of the oldest technologies used by humans, different forms of preservation were found and perfected for this purpose (Mpountoukas *et al.,* 2008). The most preservatives that commonly used are: sodium benzoate and sodium nitrite (Lennerz *et al.,* 2015). Sodium benzoate has been used as a preservative because of its good stability and solubility in water (Ren *et al.,* 2014). It is considered "Generally recognized as Safe" “GRAS” by FDA in some foods and present in foods with concentration level not exceeding 0.1 percent. Population studies indicate that soft drinks and candies are the major dietary source of benzoate (Lennerz *et al.,* 2015). The chemical industry boomed after the second world war due to enhanced cooperation within food industry. This led to the entering of artificial petroleum-based ingredients in artificial food colors, which became very popular over natural colours due to their manufacturing benefits, such as low costs and long shelf life (Martins *et al.,* 2016). Among various food additives used in meat curing, nitrite has a wide range of uses, including dye

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manufacturing and also food preservation. Nitrite in various meat products are preservative agent and inhibiter of several unwanted micro-organisms like *Clostridium botulinum* (Constanza *et al.,* 2021) .

All food additives should remain under evaluation whenever appropriate, considering the new scientific data (Gasem, 2016), these are substances capable of triggering adverse reactions, just as any other drug does, including allergic reactions, behavioral changes and carcinogenicity (Cardoso *et al.,* 2017).

The MTT assay is a sensitive, colorimetric and quantitative assay that measures proliferation, activation and viability of cells (Tim, 1983). Such assay based on the capacity of mitochondrial dehydrogenase in living cells to convert the yellow water-soluble substrate,3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyl tetrazolium bromide (MTT) into a dark blue formazan product which is insoluble in water. It is performed to measure the metabolic activity in chosen living cells. The test principle is based on the yellow colored tetrazolium salt MTT reduction to a purple formazan crystal by introducing and changing in the metabolically of active cells, and the succinate dehydrogenase system of active mitochondria is responsible for this conversion (Kalina and Palmer 1968).

The aim of this research is assess the potential cytotoxic of human lung cells caused by individually and/or combined with other food additives.

5. Material and methods

5.1 Preparation of food additives:

The chosen additives are that most commonly used as a preservatives i.e .potassium benzoate and sodium nitrite , as well as coloraning agent additives which is existed in many food categories , allura red was also chosen . The previous additives were purchased from Sigma Co., Cairo, Egypt and checked in food additives regulation (4 -2020 and 5-2023) which issued by National of Food Safety Authority (NFSA) in Egypt.

Additives were individually tested and the combination effect of them were also tested. Combination of food additives ratio was done according to their adding ratio in (4-2020 and 5-2023) additive regulation, additives were firstly weighing per mg then reweighing in ug/ml according to MTT method in Biotechnology Lab of National Research Centre, Dokki, Egypt. Each additive was tested in 6 concentrations 1000,500,250,125,62.5 and 31.25 ug/ml according to MTT test method (Mosmann 1983).

The chosen concentrations of food additives were 1000 ,500,250,125,62.5 and 31.25 ug for studying combination additives :

1-Potassium benzoate and sodium nitrite, their adding ratio was (1000: 150 mg) , according to their limit in 4-2020 legislation . then concentrations of the combination compound were prepared in the Biotechnology lab of the National Centre of Research.Dokki , Giza .Egypt.

2-Potassium benzoate and allura red, their adding ratio was (1000:300 mg), according to their limit according to (4-2020 and 5-2023) Legislation, then the concentrations of the combination compound were prepared in the Biotechnology lab. National Centre of Research, Dokki, Giza, Egypt.

Table 1 chosen food additives and their purchasing source

Additive	Source
Sodium nitrite (E250)	Sigma Company Cairo, Egypt.
Potassium benzoate (E 212)	
Allura red (E 129)	

Table 2. Tested individual additives and combined ones with their adding ratios
grape seeds extract was tested as negative control and was tested in National Research Center.

Individual tested additive	Additive percentage (mg/kg)	Combined tested additive	Additives percentage
potassium benzoate	1000	Sodium nitrite and potassium benzoate	150+1000
Sodium nitrite	150	Allura red and potassium benzoate	300+1000
Allura red	300		

5.2 Investigation of cytotoxicity effect of analysed food additives by MTT test and morphological assay:

5.2.1 MTT test

Cytotoxicity of additives was performed by MTT test on wi38 cells (human lung cells) which were purchased and prepared in the National Research Centre, Dokki, , Egypt and the test was done as follows:

Ninety nine of well tissue culture plate were inoculated with 1 X 10^5 cells / ml (100 ul / well) then incubated at 37°C for 24 hours to develop a complete monolayer sheet then growth medium was decanted from 96 well micro titer plates after confluent sheet of cells were formed. Cell monolayer was washed twice with wash media, two-fold dilutions of tested sample were made in RPMI medium with 2% serum (maintenance medium). Of each dilution, 0.1 ml was tested in different wells leaving 3 wells as control, receiving only maintenance medium. Plate was incubated at 37°C and examined. Cells were checked for any physical signs of toxicity, e.g. partial or complete loss of monolayer, rounding, shrinkage or cell granulation. MTT solution was prepared (5mg/ml in PBS) (BIO BASIC CANADA INC). MTT solution (20ul) were added to each well. Placed on a shaking table, 150rpm for 5 minutes, to thoroughly mix the MTT into the media. Incubate (37^O C, 5% CO_2) for 4 hours to allow the MTT to be metabolized. Dump off the media. (dry plate on paper towels to remove residue if necessary. Resuspend formazan (MTT metabolic product) in 200ul DMSO. Place on a shaking table (150rpm for 5 minutes) to thoroughly mix the formazan into the solvent. Presence of viable cells was visualized by the development of purple color due to formation of formazan crystals. Suspension was transferred to the cuvette of a spectrophotometer and the optical density values were measured at 620nm using DMSO as a blank. Measurements were performed and the required concentration for a 50% inhibition of viability (half maximal inhibitory concentration IC50) was graphically determined Standard graph was plotted by taking concentration of the drug in X axis and relative cell viability in Y axis, according to Pad prism program (Yifeng et al 2016). Optical density at 560nm were recorded and subtract background at 620nm. Optical density should be directly correlated with cell quantity, 3 replicates have been done and results were recorded (Tim ,1983; Ven de , 1994). The viable cells which remained after additives cytotoxicity effect (upon spectrophotometer readings) were recorded.

5.3. Statistical analyses

Data expressed as the mean values of three replicates and standard deviations were statistically analysed by performing analysis of variance technique (ANOVA) using SAS (2008).

6. Results and Discussions

6.1 Cytotoxicity effect of potassium benzoate on normal lung cells:

At the highest concentration of potassium benzoate (1000 ug/ml), the viable cells which remained were 2.37% that means the additive affected on 97.63 % of cells , the percentage of viable cells were increased by decreasing the additive concentration and the cells remain affected at 500, 250, 125 and 62.5 ug . At 31.25 ug, toxicity affected 24.57% of cells . IC_{50} of potassium benzoate is 86.9 ug , these results revealed the highly significant toxicity effect ($P<0.05$) of the additive. The effect of benzoate additive was significant in all concentrations ($P<0.05$) in all concentrations and the most significant effect of 1000 ug ($p<0.01$)

Table 3. Viability and toxicity effect of potassium benzoate on normal lung cells

Item	Concentration ug/ml	**Viability % ±SD**	**Toxicity% ±SD**	IC 50 **±SD**
Normal lung cells	100		0	ug
Potassium benzoate	1000	2.37± 0.001	97.63± 0.001	86.92 ± 0.91
	500	7.69± 0.01	92.31± 0.01	
	250	20.23± 0.03	79.77± 0.03	
	125	52.48± 0.018	47.52± 0.018	
	62.5	63.55± 0.029	36.45± 0.029	
	31.25	75.43± 0.01	24.57± 0.010	

According to the **Figure 1** , the viable cells percentage was increased at the low additive concentrations, approximately half of the cells remained at 125ug/ml concentration, 75.43% of cells remained their viability , at 31.25 ug as shown earlier. The deterioration of cells structure was very high due to the effect of benzoate on the cells.

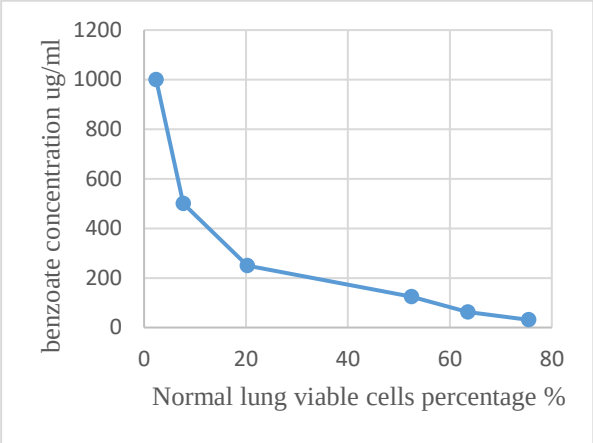

Fig 1. Toxicity effect of benzoate on normal lung cell shape.

6.2 Cytotoxicity effect of sodium nitrite on normal lung cell

Table 4. Viability and toxicity effect of sodium nitrite on normal lung cells

Item	Concentration ug/ml	**Viability % ±SD**	**Toxicity% ±SD**	IC 50 **±SD**
Normal lung cells	100			ug
Sodium nitrite	1000	1.27±0.007 98.73±0.007	1.27±0.007 98.73±0.007	82.65 ± 0.49
	500	4.71 ± 0.048	4.71 ± 0.048	
	250	10.26± 0.009	10.26± 0.009	
	125	35.64± 0.094	35.64± 0.094	
	62.5	63.26± 0.099	63.26± 0.099	
	31.25	69.36± 0.099	69.36± 0.099	

At the highest concentration of sodium nitrite (1000 ug/ml), the viable cells which remained were only 1.27% , which means that nitrite additive affected 98.73 % of cells, the percentage of viable cells have increased by decreasing the additive concentration and the viable cells remain affected by 500, 250, 125 , 62.5 and 31.25 ug. The percentage of viable cells were approximately the same in 62.5 and 31.25 concentrations. IC_{50} of sodium nitrite is 82.65 ug, these results revealed the high toxicity effect of sodium nitrite additive which is higher than that of benzoate.

The percentage of remained viable cells are 63.26 and 69.36% at 62.5 and 31.25 ug of the additive , respectively 30.64% of the cells lost their viability because of the additive effect . At the higher concentrations (1000 or 500 ug) there are high cytotoxicity effect according to the additive cytotoxicity effect on cells structure and enzyme system, but this effect decreased at low concentrations.

system, but this effect decreased at low concentrations. The effect of nitrite additive was significant in 1000 ug ($P<0.05$) , also at 500 and 250 ug the results show the significant effect of the two concentrations.

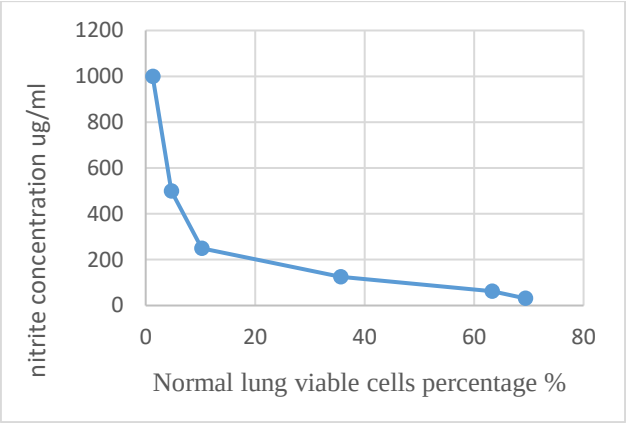

Fig 2. Toxicity effect of nitrite on normal lung cell shape

6.3 Cytotoxicity effect of Allura red on normal lung cells

The results revealed that at the highest concentration of Allura red (1000 ug/ml) , the The results revealed that at the highest concentration of allura red (1000 ug/ml) , the affected lung cells were 25.57% and the percentage of viable cells was gradually increased by decreasing the additive concentration . The 1000 ug concentration affected 74.43% of the normal lung cells, meanwhile 250 ug concentration caused 54.42% of cells to affected. At lower concentrations (62.5 and 31.25 ug) , the affected cells were 21.66 and 14.43% respectively . . Effect of allura red was significant in all concentration ($p< 0.05$). The significant effect was the highest in 1000 ug concentration ($p< 0.01$)

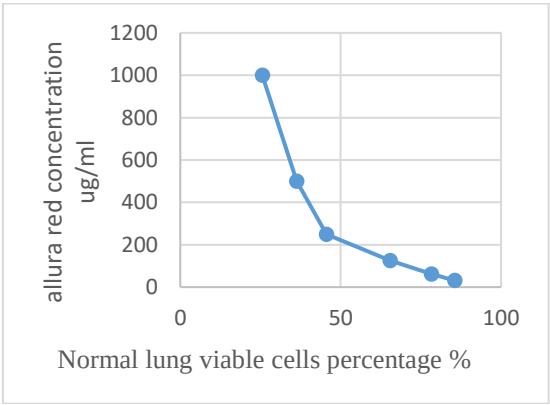

Fig 3. Toxicity effect of allura red on normal lung cells shape.

Tabel 5. Comparison between additives cytotoxicity effect on viable normal lung cells at various concentrations.

Concentration Ug/ml	Cytotoxicity of Viable cells affected by Potassium benzoate	Cytotoxicity of Viable cells affected by Sodium nitrite	Cytotoxicity of Viable cells affected by Allura red
1000	2.37	1.27	25.57
500	7.69	4.71	36.24
250	20.23	10.26	45.58
125	52.48	35.64	65.42
61.5	63.55	63.26	78.34
31.25	75.43	69.36	85.57
Ic 50	86.92	82.65	300.48

The results from **table 6** showed that all of three food additives have the ability to induce cytotoxicity in normal lung cells .They have different cytotoxicity effects on the cells. At all concentrations, sodium nitrite has the most cytotoxicity effect on the cells , sodium nitrite had the highest effect on the live cells (Table 4) in all the used additive concentrations, that due to the highly effect of the additive on NAD(P)H-dependent oxidoreductase enzymes of the cells known as mitochondrial dehydrogenase enzymes (**Revathi et al. 2023**) . The effect of potassium benzoate was similar to that of nitrite one at 61.5 ug only and lower than effect of sodium nitrite at the other concentrations. The results revealed the high toxic effect of the most two common used preservatives (nitrite and benzoate) . Allura red has lower in its cytotoxicity effect on the cells, but still there is a significant effect on the cells structure.

6.4 Cytotoxicity effect of using combined potassium benzoate and sodium nitrite on normal lung cells :

Table 6: Viability and toxicity effect of using potassium benzoate and sodium nitrite additives on normal lung cells

Item	Concentration ug/ml	**Viability % ±SD**	**Toxicity% ±SD**	IC 50 **±SD**
Normal lung cells	100			ug
Sodium nitrite and potassium benzoate	1000	1.25± 0.010	98.42± 0.010	53.54 ± 3.44
	500	3.24± 0.018	96.76± 0.018	
	250	5.78± 0.008	94.22± 0.008	
	125	26.19± 0.007	73.81± 0.007	
	62.5	32.48± 0.005	67.52± 0.005	
	31.25	42.24± 0.001	57.76± 0.001	

From **Table 6** it was proven that, the combined additives of benzoate and nitrite has a high toxic effect on normal lung cells and the combined IC_{50} is lower than that of each individual one, while at 1000 ug of the combined additives the affected cells were 98.42%, while at the least concentrations 697.52% and 57.76% of the cells were affected.

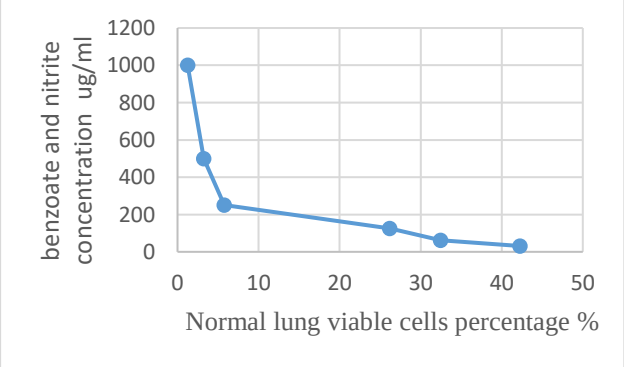

Fig 4. Viability and toxicity effect of using combined potassium benzoate and sodium nitrite additives on normal lung cells.

From **Fig 4**, the high cytotoxicity effect on the lung cells is very clear and there is high deterioration of the lung cells at concentrations of 1000, 500, 250 ug owing to the potassium benzoate and sodium nitrite combination effect on the cells, this cytotoxicity effect was still high even at the lower concentrations. At concentrations of 125, 62.5 and 31.25 ug , the viability of the cells were lower than 50% , the most of viable cells were affected even at the lowest used concentration (31.25 ug).
The results showed the significant effect of the combined additives which were ($p<0.05$) at all concentrations.

6.5. Cytotoxicity effect of combined potassium benzoate and allura red on normal lung cells

The combined additives of benzoate and Allura red had high toxic effect on normal lung cells, IC_{50} was lowest than that of each individual effect (**Table 7)**, while 1000 ug of the combined additive affected 97.72% of the normal cells and at the least concentration (31.25%), 85.01% of the cells were affected.

. The results showed the significant effect of the combined additives which were ($p<0.05$) at all concentrations.

Table 7. Viability and toxicity effect of potassium benzoate and Allura red on normal lung cells.

Item	Concentration ug/ml	**Viability % ±SD**	**Toxicity% ±SD**	IC 50 **±SD**
Normal lung cells	100			ug
Sodium nitrite and potassium benzoate	1000	2.28± 0.023	97.72± 0.023	68.8 ± 2.35
	500	3.47± 0.020	96.53± 0.020	
	250	10.69± 0.006	89.31± 0.006	
	125	21.53± 0.011	78.47± 0.011	
	62.5	42.76± 0.002	57.24± 0.002	
	31.25	85.01± 0.002	14.99± 0.002	

The cytotoxicity effect on the cells is due to the deterioration in cells enzymatic system especially at highest concentration 1000 ug/ml of combined potassium benzoate and Allura red (Özlem 2017) . At the lowest concentration, the cytotoxicity effect became lower at 31.25 ug concentration, only 14.99% of lung cells were affected and 85.01% of cells remained at the normal structures.

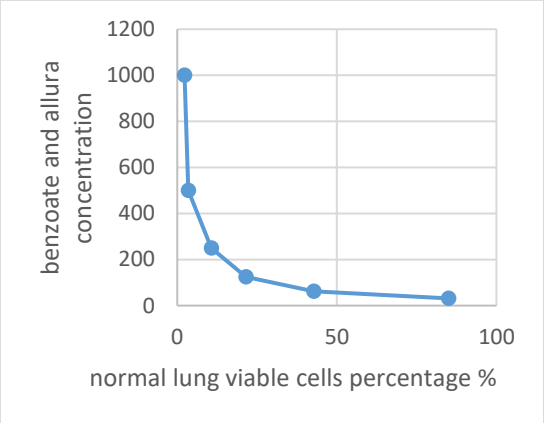

Fig 5. Viability and toxicity effect of potassium benzoate and Allura red on normal lung cells.

Tabel 8. Comparison between additives cytotoxicity effect on viable normal lung cells at various concentrations.

Concentration Ug/ml	Potassium benzoate and sodium nitrite	Potassium benzoate and Allura red
1000	1.25	2.28
500	3.24	3.47
250	5.78	10.69
125	26.19	21.53
61.5	32.48	42.76
31.25	42.24	85.01
Ic 50	53.54	68.8

Collecting data results in Table 8 revealed the high toxic effect when two combined additives were used and the toxicity was higher than that of using individual ones, The combination of benzoate and Allura red are existing in most food categories in (4-2020 legislation) and Allura red is an azo dye and found in most food products especially candies and juices. The interaction between benzoate and Allura red resulted toxicity of 97.72%, 96.56% or 89.31% of the cells at 1000,500 or 250 ug/ml, respectively. The corresponding values were 78.47%, 57.42% and 14.99 % of the cells at 125,62.5 or 31.25%. The combination of benzoate and nitrite are considered because of their common use in most preserved food products and their existence in 4-2020 legislation. The results also showed higher toxic of using combination that of individual one, the interaction between benzoate and nitrite resulted in toxicity of 98.75 %, 97.6% ,94.22%,73.81%, 67.52% or 57.76% of the cells in various six used concentrations, respectively. on 31.25 ug. More than 50% of the lung cells were affected due to such combined additives effect on the cell enzymes system and mitochondria (Özlem 2017)

6.6. Negative control results

The effect of using positive control which known as antioxident compound (grape seeds extract) where given in table (10).The extract is used to prove the viability and validity of the used cells . The grape seeds extracts are antioxidants and anticancer compounds known with its beneficial and healthy usage , the collected data in table 10. Show that , there's no effect noticed on the normal lung cells , which proves the effectiveness of the tested cell cultures . the viable cells remained its viability which was above 99% in all grape seeds extract concentrations .

Tabel 9. Effect of using negative control on the normal lung viability.

Item	Concentration ug/ml	**Viability % ±SD**	**Toxicity% ±SD**
Normal lung cells		100	0
Grape seeds extract Positive control	1000	99.23± 0.032	0.766± 0.014
	500	99.171± 0.012	0.829 ± 0.025
	250	99.2445± 0.013	0.7555± 0.024
	125	99.2404± 0.045	0.7596± 0.004
	62.5	99.24± 0.032	0. 76± 0.003
	31.25	99.292± 0.015	0.708± 0.025

7. Conclusion

It can be concluded that random usage of additives must be avoid, but under scientific assessment studies. The local products especially that consumed by children must taken under consideration and the random adding of coloring agent and/or preservatives must be put in mind to avoid the cytotoxicity effect of the combined use of them which is significantly more than that of individual effect of them .The control measures must be applied on the markets to forbidden the random adding of the additives, also adding preservatives to processed meat products need more studying and must be limited .

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