

การศึกษาฤทธิ์การก่อกลายพันธุ์และการปรับเปลี่ยนฤทธิ์ก่อกลาย
พันธุ์ของยูรีเทนของสารผสมระหว่างโซเดียมเบนโซเอทและวิตามินซี
ภายใต้สภาวะการให้ความร้อนและ/หรือแสง

The Mutagenicity of a Solution of Sodium Benzoate and Vitamin C
under Heat and/or Light Treatment and Their Modulating Effect
on the Mutagenicity of Urethane

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บทคัดย่อ

สารละลายของโซเดียมเบนโซเอท (1.4-5.6 mM) และวิตามินซี (0.7-2.8 mM) ที่ความเข้มข้นต่างๆ กัน จำนวน 9 ตัวอย่างและเครื่องดื่มที่ใช้สารเจือปนทั้งสองจำนวน 4 ตัวอย่าง ได้รับความร้อน 45 องศาเซลเซียส นาน 20 ชั่วโมง หรือ บรรจุตัวอย่างในขวดใสหรือขวดทึบแสงแล้วตากแดดนาน 8 ชั่วโมง ถูกทดสอบความเป็นพิษ และฤทธิ์ก่อกลายพันธุ์ต่อหนอนแมลงหวี่ (*mwh flr⁺/mwh TM3*) อายุ 3 วัน ที่เลี้ยงในอาหารทดลองที่มีตัวอย่าง โดยผลการศึกษาพบว่า ทุกตัวอย่างไม่แสดงฤทธิ์ก่อกลายพันธุ์ คาดว่าเนื่องจากปริมาณของสารที่เกิดขึ้น (เบนซีน) มี ปริมาณไม่สูงพอในการก่อกลายพันธุ์ในหนอนแมลงหวี่ ในกรณีให้หนอนแมลงหวี่ได้รับตัวอย่างสารละลายของ โซเดียมเบนโซเอทและวิตามินซีร่วมกับยูรีเทน (20 mM) พบว่าตัวอย่างสามารถลดฤทธิ์การก่อกลายพันธุ์ได้ คาดว่าเบนซีนที่เกิดขึ้นยับยั้งการทำงานของ CYP2E1 ในระบบกระตุ้นการก่อกลายพันธุ์ของยูรีเทน อีกทั้งเครื่องดื่มทั้ง 4 ตัวอย่างเมื่อได้รับความร้อนสามารถเพิ่มฤทธิ์การก่อกลายพันธุ์ของยูรีเทน อาจเป็นเพราะตัวอย่างทำจากน้ำส้มเข้มข้นที่มีฟลาโวนอยด์ซึ่งบางชนิดเป็นสารก่อกลายพันธุ์ธรรมชาติ ฤทธิ์การก่อกลายพันธุ์ในเครื่องดื่มที่ตรวจสอบพบในการศึกษานี้แนะนำให้ควรเก็บเครื่องดื่มในสถานที่เหมาะสมทั้งด้านอุณหภูมิและแสง

Abstract

Nine solutions composed of sodium benzoate (1.4-5.6 mM) and vitamin C (0.7-2.8 mM) as well as four soft drinks containing both food additives were heated at 45°C for 20 hr or exposed to sunlight for 8 hr in either amber or transparent glass bottles. The water in standard fruit fly medium was replaced with each sample to create the experimental medium. Three-day old *mwh+/+flr³* larvae were transferred to each

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experimental medium (mutagenicity evaluation) or an experimental medium that had 20 mM urethane (modulating effect on the mutagenicity of urethane study). All samples were not mutagenic; this possibly may be due to benzene formed in the reaction not being enough to express its mutagenicity. Each sample administered simultaneously with urethane slightly reduced the number of induced wing spots, suggesting that the product competitively inhibited CYP2E1 of phase 1 biotransformation. It is worth noting that enhancement of mutagenicity of urethane by heated commercial soft drinks is due the mutagenicity of some flavonoids in the orange juice concentrate used in preparation of each commercial beverage. The mutagenicity enhancing effect of orange juice detected in this experiment suggests that orange juice should be protected from light and heat during storage.

คำสำคัญ: โซเดียมเบนโซเอท วิตามินซี การก่อกลายพันธุ์

Keywords: Vitamin C, Sodium benzoate, Mutagenicity

Introduction

Several studies have investigated the presence of benzene in food (McNeal et al., 1993) mostly in beverages (Page et al., 1992; Nyman et al., 2008). Lachenmeier et al. (2008) revealed the formation of benzene in carrot juice. It was predominantly caused by a heat-induced mechanism. McNeal et al. (1993) reported the level of benzene found in foods containing sodium benzoates in addition to vitamin C ranged from less than 1 to 38 ng/g. Both substances are often used in beverage formulations (Ashurst, 2005). These findings were similar to survey results reported by Page et al. (1992) for benzene residues in beverages. Soft drink companies have not publicly declared the documentation of benzene contamination in their soft drinks. It was originally thought that the contaminant was caused by contaminated carbon dioxide, but some research reported that sodium benzoates and vitamin C could react to produce benzene (Chang and Ku, 1933; Gardner and Lawrance, 1993). This very harmful to consumer's health since benzene is a carcinogen (Health Canada, 2006).

During summer in Thailand, soft drinks may be exposed to sunlight during transportation or improper storage. However, it is surprising that there is no report of the presence of benzene in soft drinks sold in this country. Therefore, it was of interest to reveal the possible mutagenicity of the heat treated solution of benzoate salt and vitamin C as well as the commercial soft drinks containing both the additives. The somatic mutation and recombination test (SMART) using *Drosophila melanogaster* was a method of choice in order to justify the possible existence of a mutagen, possibly benzene, in such samples.

Materials and Methods

Chemicals: Sodium benzoate was obtained from BDH Chemical Ltd. (Poole, England) while vitamin C was purchased from E. Merck (Darmstadt, Germany). Urethane (URE) was purchased from Sigma Chemical (St. Louis, Mo, U.S.A.). Glycerol was bought from Famitalia Carlo Erba (Milan, Italy). Choral hydrate was supplied by Srichand United Dispensary Co., Ltd. (Thailand). Propionic acid was purchased from Fluka Chemika (Buchs, Switzerland).

All chemicals were of laboratory grade.

Sample Preparation The first group of samples consisted of two commercial soft drinks containing unknown amounts of sodium benzoate and vitamin C as indicated on their labels and another two soft drinks which had the same formulae that

contained sodium benzoate and vitamin C as declared in the web site of US.FDA (2007) and which were purchased locally. The second group was the solution of sodium benzoate and vitamin C at various concentrations and was prepared as indicated in Table 1.

Table 1. Solution of sodium benzoate and vitamin C. Equal volume of each solution was mixed to obtain the final concentration as indicated below.

Sodium benzoate (mM)	Vitamin C (mM)		
	0.7	1.4	2.8
1.4	Solution 1	Solution 2	Solution 3
2.8	Solution 4	Solution 5	Solution 6
5.6	Solution 7	Solution 8	Solution 9

The first portion (100 ml) of each sample was kept tightly in a 250 ml plastic screw cap Erlenmeyer flask in darkness as a control. The second portion (100 ml) of each sample was heated at 45°C for 20 hr in a water bath following McNeal et al. (1993). The third portion (100 ml) of each sample was measured into a transparent glass bottle and an amber glass bottle; then, each was exposed to direct sunlight in April 2009 (in order to mimic transportation of soft drinks in a hot climate) for 8 hr (8.00 a.m. to 4.00 p.m.).

Mutagenicity Testing Each sample was substituted for distilled water in the standard *Drosophila* medium described by Roberts (1986) to obtain each experimental medium; it was used to determine the mutagenicity of each sample. Standard medium was used as a negative control while standard medium containing urethane (20 mM) was used as a positive control. An experimental medium containing urethane was prepared by substituting each sample containing 20 mM URE for distilled water. It was used in the study to monitor the modulating effect

of each sample on the mutagenicity of URE. Equal amounts of each medium were transferred into a 15-ml test tube. The mutagenicity of each sample was assayed as described by Graf et al. (1984). The surviving adult flies bearing the marker *trans*-heterozygous (*mwh+/+flr³*) indicated with round wings were collected. The wings were removed, mounted and scored under a compound microscope for recording of the wing spots. An induction frequency of wing spots of each treated group was statistically compared with that of the deionized water negative control group as described by Frei and Wurgler (1988).

The modulating effect of each sample was estimated as follows: percentage of modulation = $(a-b)/a \times 100$. Where “a” is the number of total spots per wing induced by URE, “b” is the number of total spots per wing induced with urethane administered with each sample. It is proposed that percent of modulating effect between 0-20%, 20-40%, 40-60% and higher than 60% would indicate negligible, weak, moderate and strong, respectively.

Results and Discussion

1. Mutagenicity of Samples and Survival of Adult Flies

Since the survival rate and size of adult flies obtained from larvae fed on medium containing each sample presented in Figures 1 and 2 was not different from that of the control, it indicated the safety of samples. It seemed that the concentration

of the reaction between sodium benzoate and vitamin C, possibly benzene, might not be high enough to express its mutagenicity in the tester organism. The reason that the tester flies could effectively get rid of the product obtained from the reaction might be due to that *Drosophila melanogaster* is an organism that has high bioactivation of promutagen (Frölich and Würigler, 1989) as well as it should have a prompt corresponding conjugating system of phase 2.

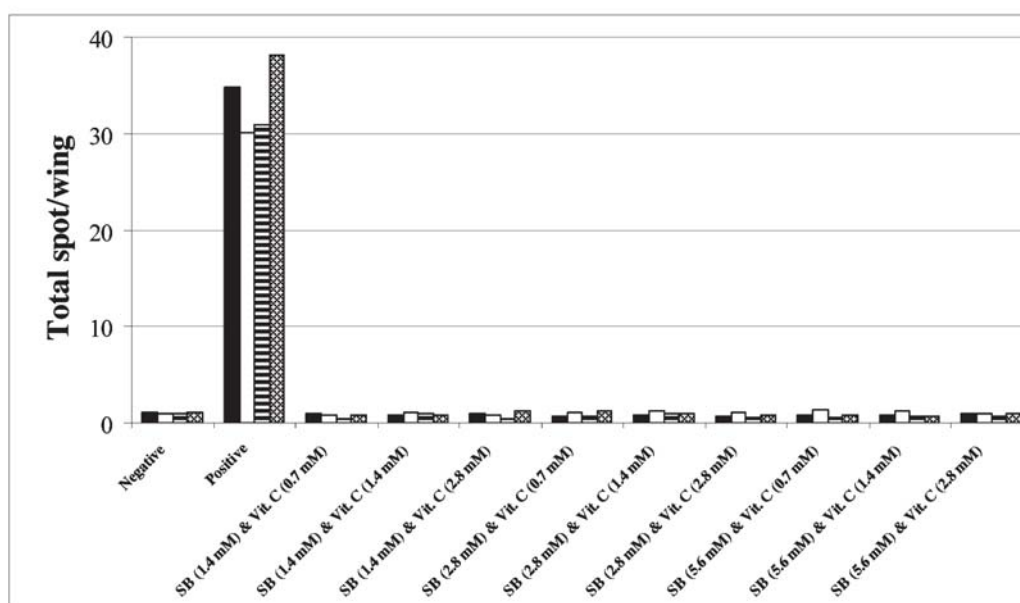


Figure 1. Mutagenicity of each solution of sodium benzoate (SB) and vitamin C stored at room temperature (■), heated at 45 °C, 20 hr in a water bath (□), exposed to sun light in a transparent glass bottle, 8 hr (▨) and exposed to sun light in an amber glass bottle, 8 hr (▩). The results are reported as wing spot induction on *Drosophila melanogaster*

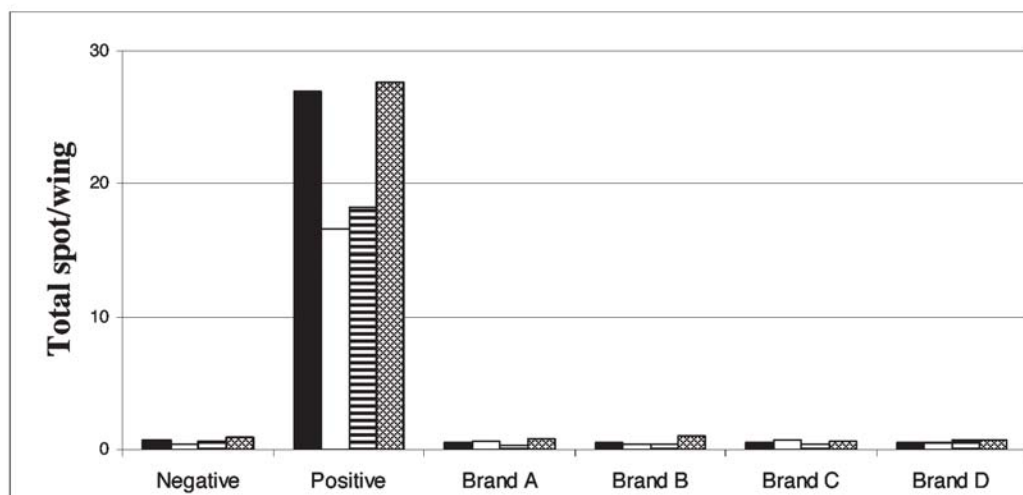


Figure 2. Mutagenicity of each commercial soft drink stored at room temperature (■), heated at 45 °C, 20 hr in a water bath (□), exposed to sun light in a transparent glass bottle, 8 hr (▨) and exposed to sun light in an amber glass bottle, 8 hr (▩) The results are reported as wing spot induction on *Drosophila melanogaster*

2. Modulating Effect of Sample on Mutagenicity of Urethane

The aim of co-administration of urethane and each solution of sodium benzoate and vitamin C treated under a specific condition was to elucidate whether each sample could modify the mutagenicity of urethane. Most samples weakly or moderately reduced the mutagenicity of urethane; however some demonstrated an enhancement effect at a negligible level (Figure 3). It is suggested that the sample might act as a competitive inhibitor of the cytochrome P450 system. About 0.5% of urethane is metabolized by CYP2E1 to vinyl carbamate which is converted by epoxidation to the putative ultimate mutagen vinyl carbamate epoxide (Miller and Miller, 1983;

Guengerich and Kim, 1991) that covalently bind to DNA, RNA and proteins to form adducts. However, it is also known that the biotransformation of benzene also requires phase 1 enzymatic reaction of CYP2E1 to transform it to its intermediate metabolite namely, hydroquinone and catechol (Seaton et al., 1994). Therefore, the number of active sites of CYP2E1 required for the activation of urethane might be reduced. The intermediate metabolites of benzene, are conjugated via a phase 2 enzymatic reaction and are excreted as sulfate and glucuronide conjugates (Seaton et al., 1995; Falany, 1991) which is different from that of urethane (vinyl carbamate epoxide) that is further conjugated with glutathione-S-transferase (GST) (Kemper et al., 1995).

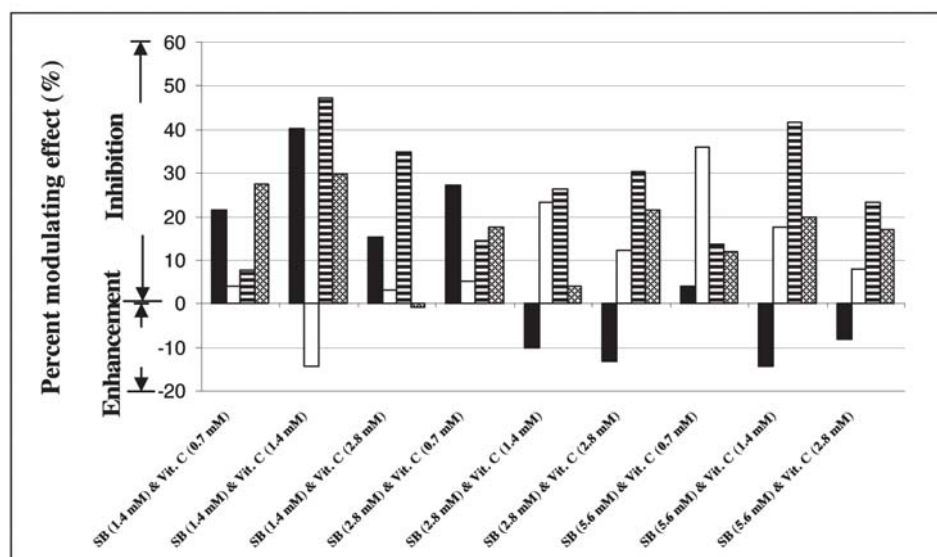


Figure 3. Modulating effect of each solution of sodium benzoate (SB) and vitamin C stored at room temperature (■), heated at 45 °C, 20 hr in a water bath (□), exposed to sun light in a transparent glass bottle, 8 hr (▨) and exposed to sun light in an amber glass bottle, 8 hr (▩) on urethane (20 mM) induced somatic mutation and recombination in *Drosophila melanogaster*

Since soft drink was stored tightly in the original container and kept at room temperature, vitamin C that was an additive in each soft drink was not involve in the reaction to form benzene. Vitamin C itself has antioxidant activity and acts as an antimutagen (Ghaskadbi and Vaidya, 1989) through multiple inhibitory mechanisms (De Flora S and Ramel, 1988). Incorporating each soft drink to the fly medium in this experiment, vitamin C might act as a free radical scavenger (Bala and Grover, 1989) in diminishing the free radicals generated during the metabolism of urethane. It was documented that *N*-hydroxyurethane, a urethane metabolite (Boyland and Nery, 1965; Nery, 1968), was hydrolyzed by esterase to generate hydroxylamine and exerted its mutagenic effect in multiple organs via generating $O_2^{\cdot-}$ and NO^{\cdot} to cause oxidation and depurination of DNA (Sakano et al., 2002). The soft drinks kept at room temperature might reduce $O_2^{\cdot-}$ and/or NO^{\cdot} in

urethane metabolism with variable degrees. Antioxidants have been suggested to scavenge free radicals, and prevent their interactions with cellular DNA (Ferguson et al., 2004).

3. Enhancing the Mutagenicity of Urethane by Commercial Soft Drinks Treated Under a Specific Condition

Heating under various conditions, the soft drinks had a tendency to increase the mutagenicity of urethane. The soft drink C (heated at 45 °C, 20 hr in a water bath) and D (heated under sunlight in an amber glass bottle) enhanced the mutagenicity of urethane at a moderate level (Figure 4). Although most soft drinks might contain both sodium benzoate and vitamin C, the concentrations should not as high as those of the nine tested solutions, thus no competitive inhibition occurred in this part of the study.

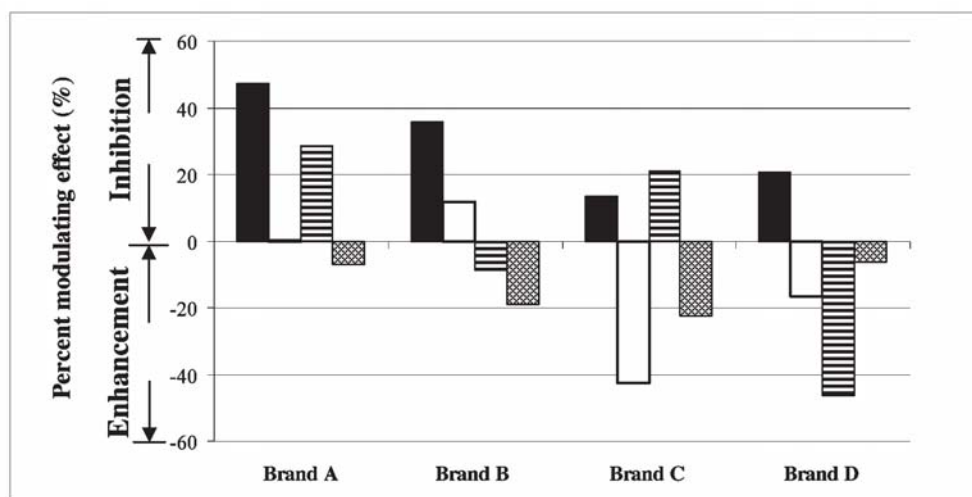


Figure 4. Modulating effect of each commercial soft drink stored at room temperature (■), heated at 45 °C, 20 hr in a water bath (□), exposed to sun light in a transparent glass bottle, 8 hr (▨) and exposed to sun light in an amber glass bottle, 8 hr (▩) on urethane (20 mM) induced somatic mutation and recombination in *Drosophila melanogaster*

Enhancement of the mutagenicity of urethane in soft drinks that were heated might be due to the fact that 3 of 4 soft drinks were made of orange juice concentrates. They were Brand (A) containing caffeine and soda pop, (B) containing 10% concentrated orange juice, (C) containing 25% concentrated orange juice, and (D) containing 5% concentrated orange juice. Variable amounts of natural compounds such as flavonoids (Bronner and Beecher, 1995; Mouly et al., 1998) namely, quercetin and other phenolic compounds were determined in commercial orange juices. The genotoxicity of quercetin (Sahu and Gray, 1996) and phenolic compounds (Do Céu Silva et al., 2003) was reported as a result of the production of reactive oxygen species. Both gave rise to the superoxide anion by auto-oxidation which led to the formation of H_2O_2 , which can produce the hydroxyl radicals that can induce DNA damage in the Ames test (Czeczot et al., 1990) and in human cells (Duthie et al., 1997). Therefore, such information might suggest that soft

drinks are potentially synergistic to the mutagenicity of urethane, which requires further elucidation.

Conclusion

This research was done in the wake of rising concerns regarding the presence of benzene, a known carcinogen, in beverages. The results of this study indicate that consumption of soft drink in the present study containing sodium benzoate and vitamin C should not be harmful to consumers. However, the finding that heat treated soft drinks could enhance the mutagenicity of urethane in the co-administration study of SMART suggests that storage of soft drinks in the appropriate conditions, namely protected from light and heat, is very important.

Acknowledgements

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