

RESEARCH ARTICLE

***Cleistocalyx nervosum* Extract Ameliorates Chemical-Induced Oxidative Stress in Early Stages of Rat Hepatocarcinogenesis**Sirinya Taya¹, Charatda Punvittayagul¹, Wanida Inboot², Shoji Fukushima³, Rawiwan Wongpoomchai^{1*}**Abstract**

Purpose: To study the effect of *Cleistocalyx nervosum* extract (CE) on diethylnitrosamine (DEN) and phenobarbital (PB) induced oxidative stress in early stages of rat hepatocarcinogenesis. **Materials and Methods:** Male Wistar rats were divided into 4 groups, with Group 1 as a negative control and Group 2 was a positive control receiving DEN injections once a week and PB in drinking water for 6 weeks. Two weeks before DEN initiation and PB treatment, Groups 3 and 4, were fed with 500 and 1000 mg/kg of CEs, respectively, for 8 weeks. **Results:** A number of GST-P-positive foci, preneoplastic lesions, in the liver were markedly increased in carcinogen administered rats, but was comparatively decreased in rats treated with 1000 mg/kg of CE. The CE reduced malondialdehyde in serum and in the livers of rats treated with DEN and PB. Moreover, CE significantly increased the activities of glutathione peroxidase and catalase in rat liver. **Conclusions:** CE appeared to exert its chemopreventive effects by modulating antioxidant status during DEN and PB induced early stages of hepatocarcinogenesis in rats.

Keywords: Antioxidant - *Cleistocalyx nervosum* - GST-P form - Hepatocarcinogenesis

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Introduction

Oxidative stress has long been known to be involved in the pathogenesis of age-related and chronic diseases, including cancer. It is defined as an imbalance between oxidants and antioxidants in favor of the former, resulting in an overall increase in cellular levels of reactive oxygen species (ROS) (Klaunig and Kamendulis, 2004). Oxidative DNA damage can trigger tumor initiation. ROS can affect a number of cellular processes critical in tumor development such as cell proliferation, senescence, inflammation and metastasis (Chandra et al., 2000).

Epidemiological studies have shown an inverse association between consumption of vegetables and fruits and the risk of human cancers at many sites (Reiss et al., 2012). It is believed that antioxidant properties of these plants protect cells from ROS-mediated DNA damage that can result in mutation and subsequent carcinogenesis (Lee et al., 2013). Anthocyanins give bright red, blue and purple colors to fruits and vegetables. They possess antioxidant activity, which is considered to be an important physiological function (PriorWu, 2006). Anthocyanins have also been reported to have anticarcinogenic and anti-inflammatory activity, and are associated with Phase II enzyme activation, apoptotic induction, anti-cell proliferation, anti-angiogenesis, anti-invasiveness and induction of differentiation (WangStoner, 2008).

Cleistocalyx nervosum var. *paniala* is an edible fruit found in South East Asia, including northern Thailand. It contains high amounts of phenolic compounds and anthocyanins. The aqueous extract of *C. nervosum* has been shown to have antimutagenic effects (Charoensin et al., 2012). In a previous report, the ethanol extract of *C. nervosum* decreased lipid peroxidation and increased glutathione peroxidase activity in rats (Thuschana et al., 2012).

About 60% of known chemical carcinogens have been found to exert carcinogenic potential to the livers of rodents. The rat liver medium-term bioassay for carcinogens has many advantages, including easy detection of preneoplastic enzyme-altered lesions, which are widely accepted as early indicators of neoplastic development (Ito et al., 2003). Glutathione-S-transferase placental form (GST-P) expression has been used as a marker for preneoplastic and neoplastic development in rat liver (Ketterer, 2001). The detection of GST-P-positive hepatocytes is one important tool for analyzing relevant carcinogenic or anticarcinogenic responses during the initiation and promotion stages of rat liver carcinogenesis (Ito et al., 1988). Diethylnitrosamine (DEN) is a potent hepatocarcinogen that has been used as an initiating agent in some two-stage carcinogenesis protocols for hepatocarcinogenic studies (Ramakrishnan et al., 2006). Phenobarbital (PB) is a classical non-genotoxic carcinogen

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that promotes hepatocarcinogenesis in rodents when administered subsequent to a genotoxic carcinogen like DEN (Zohny et al., 2013).

In the present study, we investigated the effect of *C nervosum* extract on DEN and PB induced oxidative stress during the early stages of hepatocarcinogenesis in rats, using GST-P positive foci as the endpoint marker.

Materials and Methods

Chemicals

3,3'-diaminobenzidine tetrahydrochloride hydrate and diethylnitrosamine (DEN) were purchased from Sigma, USA. Phenobarbital (PB) was purchased from Wako, Japan. Glutathione-S-transferase was purchased from MBL, Japan. A Vectastain® ABC kit was purchased from Vector Laboratories, USA. All chemicals and reagents used were of analytical grade.

Preparation of *Cleistocalyx nervosum*

The fruits of *C nervosum* were collected from Tambol Choeng Doi, Amphur Doi Saket, and Chiang Mai, Thailand. This plant was identified and confirmed by comparison with voucher specimens of known identities (QBG 7290, QBG 17340 and QBG 25139) in the Queen Sirikit Botanic Garden. The voucher herbarium specimen (Punvittayagul and Taya 1) was deposited at the Queen Sirikit Botanic Garden, Chiang Mai, Thailand. The pulp was manually separated from seed, weighed and stored in a freezer at -20°C until analysis.

Extraction procedure

CE was previously prepared in our group by Charoensin and his colleagues. Briefly, 100 g of fresh *C nervosum* pulp was added with 50 ml of distilled water and the mixture was then blended with a blender. The grounded mixture was next filtered with Whatman paper No.1 filter papers. The filtrate was centrifuged at 3,000 rpm for 15 minutes in order to collect the supernatant for lyophilization and the yield was consequently determined. The lyophilized CE was finally analyzed for a major component and stored under light protection condition at -20°C for further analysis. It has been reported that the percent yield by weight of CE is 8.48% of the fresh *C nervosum* pulp. Moreover, the major component in CE is cyanidin-3-glucoside at 85.5±16.5 mg/g CE (Charoensin, et al., 2012).

Experimental animals

Male Wistar rats, initially weighing 150-180 g, were obtained from the National Laboratory Animal Center, Salaya, Nakhon Pathom, Thailand. The animals were housed under standard environmental conditions at 24°C under a 12 hour dark-light cycle, and allowed free excess to drinking water and pelleted diet. The experimental protocol was approved by The Animal Ethics Committee of Faculty of Medicine, Chiang Mai University, Thailand.

Experimental design

Male Wistar rats were divided into 4 groups. Group 1, a negative control, was intraperitoneally injected with

0.9% normal saline solution 2 times on days 0 and 7, and received tap water throughout the experiment. Groups 2 to 4 were injected with 100 mg/kg bw of DEN on the same day as group 1. Two weeks after the first injection, rats were given 500 ppm of PB in drinking water for 4 weeks. Group 2, a positive control, were orally fed 4 ml/kg bw of distilled water for 2 weeks before the first injection of DEN and throughout the course of the experiment. Groups 3 and 4 were fed with 500 and 1000 mg/kg bw of aqueous extract, respectively for 2 weeks before the first injection of DEN and throughout the course of the experiment. The treatment protocol is shown in Figure 1. At the end of the study, the rats were anesthetized under diethyl ether. Serum was collected for biochemical determination. The livers were fixed in 10% formalin solution for histological evaluation and the remaining portions were frozen and stored at -80°C until analysis.

Immunohistochemical staining

Liver tissues were excised, weighed and cut into 3 pieces from left lateral, medial, and right lateral lobes. They were fixed in 10% phosphate buffered formalin, and embedded in paraffin for immunohistochemical examination of Glutathione-S-transferase placenta form (GST-P). After deparaffinization, 3 µm liver sections were immunostained for GST-P as described by Puatanachokchai et al. (2006) using rabbit anti rat GST-P (Medical and Biological Laboratories Co., Ltd., Nagoya, Japan, 1:2000 dilution, 37°C for 2 hours), biotin-labeled goat anti-rabbit IgG and an avidinbiotin complex method (ABC kit); Vector Laboratories, Burlingame, CA). 3'-3'-Diaminobenzidine was applied for final color development. GST-P positive hepatocytes comprised 20 or more cells and were counted under a light microscope.

Biochemical estimations

Use of a thiobarbituric acid reactive substance (TBARs) is a well-established assay for screening and monitoring lipid peroxidation. Levels of TBARs (Fujiwara, 2003; Suresh et al., 2013) and glutathione (AkerboomSies, 1981) were estimated in the serum and liver tissue. The activities of antioxidant enzymes, including catalase, glutathione reductase, glutathione peroxidase and heme oxygenase,

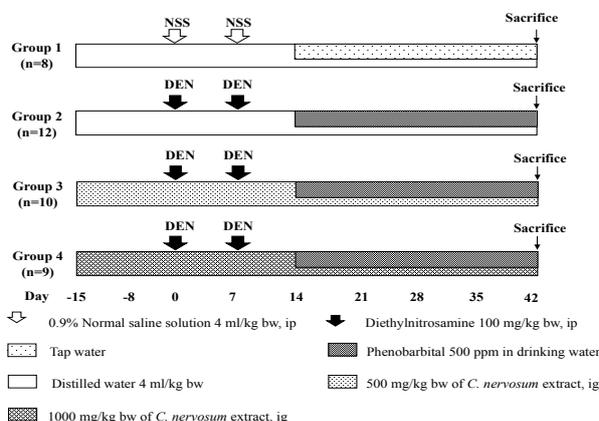


Figure 1. Treatment Protocol for the Effect of Aqueous Extract of *C nervosum* on Oxidative Stress Induced Early Stages of Rat Hepatocarcinogenesis

Cleistocalyx nervosum Extract Ameliorates Chemical-Induced Oxidative Stress in Early Stages of Rat Hepatocarcinogenesis and 530 nm.

were assayed in the liver tissues.

Catalase decomposes hydrogen peroxide into water and oxygen. Catalase activity was measured according to the method of Aebi (Aebi, 1984). The reaction mixture contained 30 mM H₂O₂, 50 mM phosphate buffer, pH 7.0 and sample in a total volume of 750 μ l. Catalase activity was estimated by the decrease in absorbance of H₂O₂ at 240 nm.

Glutathione reductase activity was determined according to the method of Carlberg and Mannervik (CarlbergMannervik, 1985). The reaction mixture contained 100 mM Potassium phosphate buffer (pH 7.0), 1.2 mM oxidized glutathione, and 1.2 mM β -NADPH. Decrease in the absorbance of β -NADPH at 340 nm was monitored spectrophotometrically, at 37°C. A unit of activity (U) was defined as the amount of enzyme that catalyzes the oxidation of 1 μ mole of NADPH in 1 minute under these conditions. Specific activity was defined as units per mg of protein.

Glutathione peroxidase activity was determined according to method described by Nagalakshmi and Prasad with slight modifications (NagalakshmiPrasad, 2001). The reaction mixture contained 0.1 M Tris-EDTA buffer (pH 8), 0.1 M reduced glutathione, 2 mM β -NADPH, 7 mM t-BHP, 10 U Glutathione reductase and 10 μ l of sample. The oxidation of β -NADPH was followed by measuring the decrease in the absorbance at 340 nm.

Heme oxygenase activity was determined according to Farombi with slight modifications (Farombi et al., 2008). The reaction mixture contained 50 μ M of the substrate hemin, microsomal fraction and rat liver cytosol as a source of biliverdin reductase, 2 mM glucose-6-phosphate, 0.2 U/ml glucose-6-phosphate dehydrogenase, 0.8 mM NADPH and 100 mM potassium phosphate buffer containing MgCl₂, pH 7.4 and was incubated at 37°C for 1 hour. The reaction was stopped with chloroform after extraction, and the chloroform layer was measured spectrophotometrically. Bilirubin formation was calculated from the difference in absorption between 460

Statistical analysis

Data are expressed as mean \pm SD of each variable for each group. The data were firstly tested for normality using a Kruskal-Wallis test. The significance of differences between groups was analyzed using the analysis of variance (ANOVA) with LSD for post hoc tests. P values<0.05 were regarded as significant.

Results

Effect of *C nervosum* extract on body and liver weights of rats treated by diethylnitrosamine and phenobarbital

Average body weights of different animal groups at various time points are shown in Figure 2. No statistical differences were observed between the growth rates of any of the treatment or control groups. Final body weights and absolute and relative liver weights of various groups are presented in Table 1. The average absolute and relative liver weights of diethylnitrosamine and phenobarbital treated rats were significantly increased as compared to those of the control group. Final body weights of diethylnitrosamine and phenobarbital treated rats were not significantly different among the groups of rats in this study. The treatment of 1000 mg/kg bw of *C nervosum* tended to reduce liver weight of diethylnitrosamine and phenobarbital treated rats.

Effect of *C nervosum* extract on numbers of GST-P positive foci in liver of rats treated with diethylnitrosamine and phenobarbital

The number of GST-P positive foci in liver of rats treated by diethylnitrosamine and phenobarbital was significantly increased as compared to that of the normal group. The *C nervosum* at a dose of 1000 mg/kg bw significantly decreased the total number of GST-P positive foci in liver of rats induced by diethylnitrosamine and phenobarbital (Table 2). The extract tended to reduce

Table 1. Body and Liver Weights of Rats Administered *C nervosum* Extract During Treatments with Diethylnitrosamine and Phenobarbital

Treatments	No. of rats	Final body weight (g)	Liver weight	
			Absolute (g)	Relative (/100 g bw)
0.9% NSS+Tap water	8	376 \pm 26	12.21 \pm 1.36	3.24 \pm 0.26
DEN+PB	12	380 \pm 31	14.91 \pm 1.66*	3.92 \pm 0.25*
DEN+PB+CE 500 mg/kg bw	10	360 \pm 32	14.51 \pm 2.07	4.01 \pm 0.26
DEN+PB+CE 1000 mg/kg bw	9	350 \pm 26	13.77 \pm 0.87	3.93 \pm 0.33

*Significant different from negative control group, p<0.05; Values are expressed as mean \pm S.D; DEN: Diethylnitrosamine; PB: Phenobarbital; CE: *C nervosum* extract

Table 2. Quantitative Data for GST-P-Positive Liver Cell Foci in rats Administered *C nervosum* Extract During Treatments with Diethylnitrosamine and Phenobarbital

Treatments	Total	Number of GST-P positive foci (foci/cm ²)		
		21-30 cells/focus	Foci containing 31-50 cells/focus	> 50 cells/focus
0.9% NSS+Tap water	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0
DEN+PB	2.64 \pm 1.04*	1.60 \pm 0.81*	0.70 \pm 0.50*	0.34 \pm 0.46*
DEN+PB+CE 500 mg/kg bw	2.87 \pm 1.92	1.78 \pm 1.26	0.84 \pm 0.88	0.25 \pm 0.35
DEN+PB+CE 1000 mg/kg bw	1.51 \pm 0.64**	1.03 \pm 0.66	0.26 \pm 0.47	0.21 \pm 0.34

*Significant different from negative control group, p<0.05; **Significant different from positive control group, p<0.05; Values are mean \pm S.D; DEN: Diethylnitrosamine; PB: Phenobarbital; CE: *C nervosum* extract

Table 3. Effect of *C nervosum* Extract on Lipid Peroxidation and Total Glutathione Content in Liver and Serum of Diethylnitrosamine and Phenobarbital Treated Rats

Treatments	TBARs (nmoles MDA formed/mg protein)		Total GSH (nmol/mg protein)	
	Serum	Liver	Serum	Liver
0.9% NSS+Tap water	12.7±1.0	66±36	6.09±2.65	6.91±3.82
DEN+PB	13.4±1.1	200±74*	5.91±2.48	2.55±1.71*
DEN+PB+CE 500 mg/kg bw	13.8±1.0	127±92	5.63±2.89	2.04±0.48
DEN+PB+CE 1000 mg/kg bw	11.3±1.0**	109±54	6.11±2.75	3.63±1.85

*Significant different from negative control group, p<0.05; **Significant different from positive control group, p<0.05; Values are mean±S.D; DEN: Diethylnitrosamine; PB: Phenobarbital; CE: *C nervosum* extract

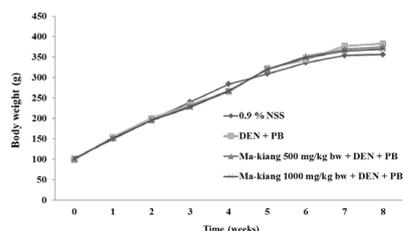


Figure 2. Growth Curve of for Rats Administered *C nervosum* Extract During Treatments with Diethylnitrosamine and Phenobarbital

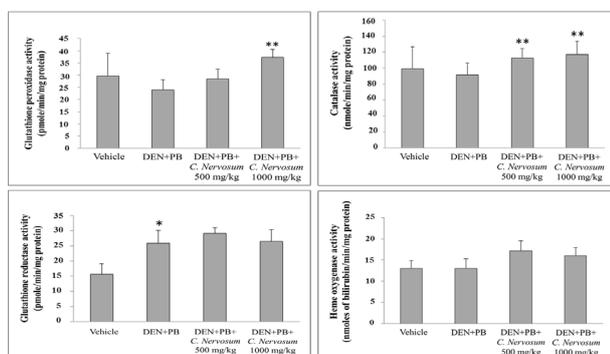


Figure 3. Effect of *C nervosum* Extract on the Activities of Antioxidant Enzymes in the Livers of Rats. *p<0.05; DEN and PB treated group as compared to negative control group; **p<0.05; 500 and 1000 mg/kg bw of *C nervosum* treated group as compared to DEN and PB treated group

medium foci and large foci rather than very large foci, which contain more than 50 GSTP labelled hepatocytes per focus.

Effect of C nervosum extract on lipid peroxidation and antioxidant enzymes in liver and serum of rats treated with diethylnitrosamine and phenobarbital

The treatments with diethylnitrosamine and phenobarbital significantly changed only the contents of TBARs and glutathione in the rat livers. The highest doses of *C nervosum* extract tended to decrease TBARs levels and increase GSH levels. Nevertheless, the level of TBARs was significantly reduced in the serum of rats treated with high doses of extract and carcinogen (Table 3).

The effect of *C nervosum* extract on antioxidant enzymes activities in rat liver is shown in Figure 3. The rats fed with *C nervosum* extract showed significantly increased glutathione peroxidase and catalase activities in the liver when compared to carcinogen treated rats. The *C nervosum* extract did not modulate the activities of glutathione reductase and heme oxygenase in the livers

of diethylnitrosamine and phenobarbital treated rats

Discussion

The treatment of *C nervosum* extract significantly decreased the number and size of GST-P positive foci in diethylnitrosamine and phenobarbital induced early stages of hepatocarcinogenesis in rats. Anthocyanins found in fruits and vegetables have been reported to possess antioxidant and anti-cancer activities. The main anthocyanins in *C nervosum* extract were cyanidin-3-glucoside, cyanidin-3, 5-diglucoside and cyanidin-5-glucoside (Charoensin et al., 2012). Cyanidin-3-glucoside had anti-tumor activity against gastric adenocarcinoma cells (Sun et al., 2012), decreased in vitro invasiveness of a human lung cancer cell line (Chen et al., 2006) and suppressed neoplastic cell transformation by directly targeting phosphatidylinositol-3-kinase (Song et al., 2012). Less is known concerning was the chemopreventive effect of anthocyanins on diethylnitrosamine and phenobarbital induced hepatocarcinogenesis in rats. Bishayee et al. reported that an anthocyanins rich-extract from black currant had chemopreventive effects against diethylnitrosamine and phenobarbital-induced hepatocarcinogenesis via inhibition of abnormal cell proliferation and induction of apoptosis (Bishayee et al., 2011).

Diethylnitrosamine, a rodent genotoxic hepatocarcinogen, could adduct DNA after being metabolized by CYP2E1, leading to fixed DNA mutation. The hepatic GST-P positive foci, a preneoplastic lesion of rat hepatocarcinogenesis in diethylnitrosamine initiated rats, are commonly detected approximately 4 weeks after a single injection (Tsuda et al., 2010). Repeated exposures to overdoses of phenobarbital, a classical non-genotoxic carcinogen in rodents, could accelerate tumor promotion by CYP2B1 mediating reactive oxygen species (Kinoshita et al., 2002; Imaoka et al., 2004; Puatanachokchai, Kakuni et al., 2006). In addition, administration of diethylnitrosamine has been reported to generate reactive substances of lipid peroxidation (Thirunavukkarasu et al., 2001; Pracheta et al., 2011), and phenobarbital enhanced the formation of reactive oxygen species in the preneoplastic nodules in rat liver (Jeyabal et al., 2005). Lipid peroxidation is considered as one of mechanisms of tissue damage caused by free radicals. An administration of diethylnitrosamine and phenobarbital has been reported to generate lipid peroxidation in the preneoplastic nodules in rat liver (Scholz et al., 1990; Singh et al., 2004). We also found increased TBARs

formation and decreased glutathione content in livers of carcinogen treated rats. The treatment with *C nervosum* extract in diethylnitrosamine and phenobarbital treated rats modulated TBARs and glutathione levels. Prior and Wu reported that anthocyanins presented radical scavenging activity (PriorWu, 2006) and Thoppil et al. also reported that an anthocyanin-rich extract from black currant exerted chemopreventive action against diethylnitrosamine-induced hepatocarcinogenesis by attenuating oxidative stress through activation of Nrf2 signaling pathway (Thoppil et al., 2012). In addition, our group found that *C nervosum* acted as an antioxidant scavenger (Thuschana, et al., 2012) and antioxidant enzyme enhancer (Taya et al., 2009; Thuschana, et al., 2012). Therefore, *C nervosum* extract containing anthocyanins might modulate oxidative status via acting as antioxidant scavenger and antioxidant enzyme enhancer in rat liver.

Phenobarbital, a tumor promoter, has a strong inhibiting effect on cellular antioxidant defense system components such as superoxide dismutase, catalase and glutathione (YadavBhatnagar, 2007). Superoxide dismutase accelerates transformation of superoxide radicals to hydrogen peroxide, while catalase or glutathione peroxidase converts hydrogen peroxide to water. Depletion in activity of these antioxidant enzymes can be due to enhanced radical production during diethylnitrosamine and phenobarbital metabolism. In the present study, hepatic TBARs levels increased in carcinogens treated rats, which were presumably associated with increased free radicals, confirming the suggestion that these free radicals inhibited the activities of superoxide dismutase, catalase, and glutathione peroxidase. The superoxide radical itself is also capable of inhibiting the activity of superoxide dismutase and catalase. The observed reduction in the enzyme activities may be attributed to reactive oxygen species (Jeyabal et al., 2005). The increased hepatic TBARs level in carcinogen treated rats was indicative of increased oxidative stress. The decreased catalase and glutathione peroxidase activities in carcinogen treated rats indicated alterations in anti-oxidant enzymes under conditions of oxidative stress. The *C nervosum* extract significantly increased catalase activity in carcinogen treated rats, while only doses of 1,000 mg/kg bw significantly increased glutathione peroxidase activity. The recovery of catalase and glutathione peroxidase activities may also be due to the anti-oxidant function of the *C nervosum* extract. During hydrogen peroxide scavenging, glutathione reduced form (GSH) is oxidized to glutathione oxidized form (GSSG) by glutathione peroxidase. The conversion of GSSH to GSH is catalyzed by glutathione reductase. However, the activity of glutathione reductase was significantly increased in carcinogen treated rats. In carcinogen treated rats, the increased glutathione reductase activity might have resulted in the reduction of glutathione peroxidase activity and content of glutathione reduced form. Thus, the chemopreventive effect of *C nervosum* extract containing anthocyanins might be due to its action as an antioxidant enhancer. Heme oxygenase-1 is a stress-responsive enzyme widely distributed in many mammalian tissues and is responsible for the breakdown of heme to biliverdin,

free iron and carbon monoxide (MainesGibbs, 2005). Heme oxygenase-1 expression is induced by a wide variety of stimuli including heme, heavy metals, cytokines and chemical carcinogens (McNally et al., 2006). Moreover, heme oxygenase-1 has been considered to be a potential therapeutic target for a number of chemopreventive agents (Prawan et al., 2005). However, heme oxygenase activity was unchanged across treatment groups in the present study. *C nervosum* extract may exert its chemopreventive effect via glutathione dependent enzymes.

Our findings indicated that *C nervosum* extract decreased preneoplastic lesions of chemically-induced rat hepatocarcinogenesis by reduction of lipid peroxidation and increase of some antioxidant markers. Hence, the chemopreventive effect of *C nervosum* might be due to either its radical scavenging properties or to antioxidant enzyme induction.

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