Full Length Research Paper

Antioxidative Efficacy of Combined Leaves Extracts of Gongronema Latifolium and Ocimum Gratissimum on Streptozotocin-Induced Diabetic Rat Models

*I. F. Usoh and H. D. Akpan

Department of Biochemistry, Faculty of Basic Medical Sciences, University of Uyo, Nigeria.

Abstract

This study investigated the effect of oral administration of combined 80% ethanolic leaves extracts of Gongronema latifolium (GL) and Ocimum gratissimum (OG) on some oxidative stress indices in diabetic rats. Thirty-six male albino rats were divided into 6 groups with 6 rats in each. Groups A and F received placebo treatment and served as diabetic and normal controls respectively. B and C received extracts of GL and OG at 200mg/kg b.w respectively. D received combined extracts of GL and OG at 100mg/kg b.w each and E received 5 IU/kg b.w insulin subcutaneously. Treatment lasted for 28 days after which animals were sacrificed and serum and liver collected for analysis of antioxidant parameters. From the results, the single and combined extracts treatment significantly (P<0.05) decreased hepatic MDA formation, suggesting the role of the extracts in protection against pro-oxidant induced membrane damage. The extracts also significantly increased the hepatic activities of SOD, GPX, CAT and serum levels of β-carotene, Vitamins A and C. The combined extracts group displayed a more pronounced effect when compared to single extract and insulin groups. The antioxidant effectiveness of the combined extracts might be connected with the synergistic interactions of several bioactive principles present in these leaves.

Keywords: Malondialdehyde, antioxidant enzymes, streptozotocin, diabetes, rats, Gongronema latifolium, Ocimum gratissimum.

INTRODUCTION

Diabetes mellitus is a metabolic disease characterized by persistent hyperglycemia, glycosuria, increased thirst (polydipsia), polyuria, polyphagia, and weight loss due to absolute or relative lack of insulin (Aguwa, 1996). Free radicals have been described as any chemical species not capable of independent existence and which contains one or more unpaired electrons. Having unpaired electron(s), they are unstable and highly reactive as the attempt is to satisfy their outer electron octet. By so doing, they abstract electrons from neighboring proteins, lipids, carbohydrates and nucleic acids setting in place a chain of reactions that lead to the cell membrane, organelles and DNA damage. Free radicals, including the superoxide radical (O$_2^-$), hydroxyl radical (OH), hydrogen peroxide (H$_2$O$_2$) and lipid peroxide radicals have been implicated in a number of disease processes, including diabetes, asthma, cancer, obesity, cardiovascular, gastrointestinal and liver diseases, cataract, macular degeneration, periodontal disease, and other inflammatory processes. These reactive oxygen species (ROS) are normal consequence of biochemical processes formed in the mitochondria and endoplasmic reticulum of aerobic organisms as oxygen is being reduced along the electron transport chain in the body, and also as a result of increased exposure to environmental and/or dietary xenobiotics (Cook and Samman, 1996; Allan and Miller, 1996; Kumpulainen and Salonen, 1999). ROS are increased in various tissues in diabetic condition and are involved in the development of diabetic complications (Brownlee, 2001). Several hypotheses put forth to explain the genesis of free radicals in diabetes include auto-oxidation processes of glucose, the non-enzymatic and
progressive glycation of proteins with the consequently increased formation of glucose-derived advanced glycosylation end products (AGEs), and enhanced glucose flux through the polyol pathway (Tiwari and Rao, 2002). The increase in ROS themselves has strongly been correlated with chronic hyperglycemia of diabetes (Kaneto et al., 2005). Hence, therapy using free-radical scavengers (antioxidants) has potential to prevent, delay or ameliorate many of these disorders (Delanty and Dichter, 2000). The antioxidant defense system represents a complex network of interactions, synergy and specific tasks for a given antioxidant (Polidori et al., 2001). The efficiency of this defense mechanism is altered in diabetes and, therefore, the ineffective scavenging of free radicals may play a crucial role in determining tissue damage (Wohaieb and Godin, 1987).

Many medicinal plants including Gongronema latifolium and Ocimum gratissimum have been reported to possess free radical scavenging (antioxidant) properties (Ugochukwu et al., 2003; Ogundipe et al., 2003, Trevisan et al., 2006, Akinmoladun et al., 2007). They contain several different pharmacologically active compounds that may act individually, additively or synergistically to improve health (Azarzeh et al., 2003). Gongronema latifolium Bent Hook, is a herbaceous shrub, with yellow flowers and the stem that yields characteristics milky exudates when cut. It is locally called “utasi” by the Efiks, Ibibios and Quas; “utazi” by the Igbos and “arokeke” by the Yorubas in Nigeria. The Efiks and the Quas in Calabar use G. latifolium crude leaf extract in the treatment of malaria, diabetes, hypertension, and as laxative. The use of crude leaf extract of this shrub in maintaining a healthy blood sugar levels has been reported (Okafor and Ejofo, 1996). Scientific studies have established the hypoglycaemic, hypolipidaemic and antioxidative effects of aqueous and ethanol extracts of G. latifolium leaf (Ugochukwu et al., 2003; Ogundipe et al., 2003). Morebise et al. (2002) showed that the leaf extract has anti-inflammatory activity with its potential nutritional and food processing properties. Some phytochemicals such as B-sistosterol, lupenyl esters, pregnane ester, glycosides, essential oils and saponins are associated with parts of this herb (Schneider et al., 1993; Morebise et al., 2002). It is plausible that one or more of these phytochemicals that are found in G. latifolium is likely to influence cellular proteins with enzymatic activity.

Ocimum gratissimum (Labiatae) is a native of Africa and Asia but is now distributed to other parts of the World including the United States of America (Sulistiarini, 1999). In Nigeria, O. gratissimum is described by different local names: Daidoya (Hausa), Nchunwu (Igbo), Efnrin (Yoruba), Nton (Ibibio) (Owulade, 2004) but it is popularly known as “scent leaf” in most parts of the country. The plant is used as a condiment and spice in most parts of the World including Nigeria for preparation of different dishes. It is also used widely in folk medicine for the treatment of several ailments including fever, cough and respiratory disorders (Correa, 1932; Oliver, 1980), sore throat, kidney stones, epilepsy and dermatitis (Sofowora, 1993), headache, stress and mental diseases (Osifo, 1989). Studies have shown that the leaf extract of O. gratissimum contains potent bioactive components (essential oils) made up of eugenol, citral, linalool, charvicol, thymol, geranol, triterpenoids, saponins, alkaloids, etc. (Sulistiarini, 1999; Leal et al., 2006; Matasyoh et al., 2007). These phytochemicals possess antibacterial (Akinyemi et al., 2005), antifungal (Dubey et al., 2000) antinoceptive (Rabelo et al., 2003), antihypertensive (Interaminense et al., 2005, 2007), anti-diabetic (Mohammed et al., 2007), antidiarrheal (Ilori et al., 1996; Adebolu and Salau, 2005), antioxidant (Odukoya et al., 2005), insecticidal (Eze et al., 2006) and antimelinctic properties which justify its high medicinal use in folk medicine (Pessoa et al., 2002).

Polyherbal therapy allows for combination of secondary metabolites which would not only exert a combined effective action in various mechanistic targets but also potentiate maximum therapeutic efficacy with minimum side effects (Tiwari and Rao, 2002; Ebong et al., 2008). From the foregoing, this study is aimed at investigating the combined antioxidant action of the ethanolic leaves extracts of Gongronema latifolium and Ocimum gratissimum in streptozotocin-induced diabetic Wistar rats.

MATERIALS AND METHODS

Collection and preparation of plant materials

Fresh but matured leaves of Gongronema latifolium and Ocimum gratissimum were collected from Atimbo, Akpabuyo Local Government Area of Cross River State. They were both identified and authenticated in the Department of Botany, University of Calabar, Calabar. 500g each of Gongronema latifolium and Ocimum gratissimum were thoroughly washed with clean tap water to remove dust particles, and debris and shade dried. The dried plant materials were separately ground and preparation of plant material.

filtration using Whatman No.1 filter paper. The filtrates were separately concentrated in vacuo at 37-40°C using a rotary evaporator. The concentrates were allowed open in a water bath (40°C) for complete ethanol removal. The dried extracts were refrigerated at 2-8°C until required for use. The concentration of the extract was determined by drying a known volume and measuring the dry weight.
**Table 1. Experimental Design**

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of animals</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>6</td>
<td>Placebo (Diabetic Control)</td>
</tr>
<tr>
<td>B</td>
<td>6</td>
<td>GL extract (200mg/kg bw)</td>
</tr>
<tr>
<td>C</td>
<td>6</td>
<td>OG extract (200mg/kg bw)</td>
</tr>
<tr>
<td>D</td>
<td>6</td>
<td>GL (100mg/kg) + OG (100mg/kg)</td>
</tr>
<tr>
<td>E</td>
<td>6</td>
<td>Insulin (5 IU/kg bw)</td>
</tr>
<tr>
<td>F</td>
<td>6</td>
<td>Placebo (normal control)</td>
</tr>
</tbody>
</table>

**Experimental animals**

Thirty-six (36) male albino rats of Wistar strain weighing between 164-258g were obtained from the animal house of the College of Health Sciences, University of Calabar. The animals were allowed to acclimatize for two weeks in the Biochemistry departmental animal house facility, University of Calabar where experiment was carried out. The animals were housed in well ventilated cages (wooden bottom and wire mesh top) where bedding was replaced every two days, and kept under controlled environmental conditions (room temperature of about 27°C and 12 hour light/dark cycle). The animals were fed with grower’s marsh and water from tap *ad libitum*.

**Induction of experimental diabetes**

Prior to diabetes induction, the rats were subjected to 12 hour fast and then diabetes was induced by intraperitoneal injection of 65mg/kg b.w (Ugochukwu and Babady, 2003) streptozotocin (STZ) (Sigma St. Louis, MO, USA) reconstituted in 0.1M Na citrate buffer (pH 4.5). Seven days after, diabetes was confirmed in STZ treated rats with a fasting blood sugar concentration ≥ 200mg/dl.

**Experimental design and treatment of animals**

The diabetic rats (n = 30) were divided randomly into five groups of 6 rats each as shown in table 1. The plant extracts reconstituted in distilled water (vehicle) were administered via oral gastric intubation at a dose of 200mg/kg body weight daily for single extract treatment and 100mg/kg body weight each in combined extract treatment twice per day (7.00am and 7.00pm). Insulin (5IU/kg body weight) was administered subcutaneously (S.C) once daily postprandial. The dosages of plant extracts and insulin used were according to the methods of Ebong et al., (2006) and Sonia and Scrinvasan, (1999). The normal control group, F (n=6) was fed with grower’s marsh and water without treatment. Treatment lasted for 28 days.

**Preparation of serum and microsomal fractions of liver homogenate**

After 28 days of treatment, the animals were anaesthetized with chloroform vapor, quickly brought out of the jar and sacrificed. Whole blood was collected by cardiac puncture from each animal using sterile needle to pierce through the heart. It was emptied into sterile test tubes containing no anticoagulant and allowed to stand for about 15 minutes to clot and further spun in a westerfuge centrifuge (Model 1384) at 10,000g at 4°C for 10 mins. Serum was separated from the clot with Pasteur pipette into sterile sample tubes for the determination of serum β –Carotene and vitamins A and B. The liver was immediately removed, washed in ice cold 1.15% KCl solution, blotted, weighed and homogenized in 4 volumes of the homogenizing buffer (pH7.4) using a potterelvegin homogenizer. The resulting liver homogenate was centrifuged at 105,000g for 1 hour in a BECKMAN L5 - 50B ultracentrifuge with a type 35 fixed angle rotors. Pellet microsomes were suspended in 0.25M sucrose solutions and this was stored in a frozen condition. These procedures were carried out at temperature between 0°C and 4°C so as to retain enzyme activity. The liver microsome was used to determine lipid peroxidation (LP), glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT).

**Determination of lipid peroxidation**

Lipid peroxidation in microsomes prepared from liver was estimated by thiobarbituric acid-reactive substances (TBARS) as described by the procedure of Varshney and Kale, (1990). It was determined by quantifying MDA concentrations, which was spectrophotometrically measured by the absorbance of a red-colored product with thiobarbituric acid.

**Determination of liver superoxide dismutase (SOD) activity**

SOD activity in hepatocytes was measured using assay kits (Randox laboratories, Crumlin, Ireland). The method
for SOD employs xanthine and xanthine oxidase (XOD) to generate superoxide radicals which react with 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT) to form a red formation dye (Woolliams et al., 1983). The SOD activity is then measured by the degree of inhibition of this reaction. One unit of SOD is that which causes a 50% inhibition of the rate of reduction of INT. The absorbance was spectrophotometrically measured.

### Determination of liver glutathione peroxidase (GPx) activity

GPx activity in hepatocytes was measured using assay kits (Randox laboratories, Crumlin, Ireland). The method is based on that of Paglia and Valentine (1967). GPX catalyzes the oxidation of glutathione by cumene hydroperoxide. In the presence of glutathione reductase and NADPH the oxidized glutathione (GSSG) is immediately converted to the reduced form (GSH) with a concomitant oxidation of NADPH to NADP⁺. The decrease in absorbance was spectrophotometrically measured.

### Determination of catalase (CAT) activity

Catalase (CAT) activity in hepatocytes was spectrophotometrically determined using assay kits from Bioxytech ® catalase-520™, based on Aebi (1984). The principle involves dismutation of hydrogen peroxide (H₂O₂) to water and molecular oxygen whose rate is directly proportional to the concentration of catalase. The sample containing catalase of unknown activity is incubated in the presence of a known concentration of H₂O₂, for exactly one minute, after which the reaction is stopped / quenched with sodium azide. The amount of H₂O₂ remaining in the reaction mixture is then quantified by the oxidative coupling reaction of 4-aminophenazone (4-aminooantipyreine,) and 3,5-dichloro-2-hydroxy benzenesulphonic acid (DHBS) catalyzed by a versatile enzyme, horseradish peroxidase (HRP). The color intensity of the resulting product (measured at 520nm) is directly proportional to amount of H₂O₂ remaining in reaction mixture but inversely proportional to catalase activity in the sample.

### Determination of serum β–carotene and vitamins A and C

Serum β-carotene and vitamin A were assayed according to the method of Suzuki and Katoh (1990) as described by Kokcam and Naziroglu (1999). Serum vitamin C was determined chemically according to the procedure described by Erel et al. (1997) using dinitrophenylhydrazine (DNPH).

### Statistical analysis

The results were reported as means ± SD from six repeated determinations and evaluated with the analysis of student’s t-test. Differences were considered to be statistically significant at P<0.05.

### RESULTS AND DISCUSSION

#### Malondialdehyde (MDA) levels and liver SOD, GPx and CAT activities

The results in table 2 revealed a significant significant decrease in the activities of SOD, GPx and CAT and significant increase in the concentration of MDA in hepatocytes of STZ-induced diabetic rats (DC) compared to normal control (NC) rats (p<0.05). Treatment with GL and OG extracts significantly brought down the level of MDA and increased the activities of the antioxidant enzymes when compared to the DC (p<0.05). Insulin treated group (Dᵢ) showed a significant increase in the concentration of MDA and the activities of antioxidant enzymes when compared to extract treated groups. The combined extracts treated group (DₓGLOG) exhibited a significant decrease in the concentration of MDA and increase in the activities of SOD, GPx and CAT when compared to single extract treated groups.

#### Serum β-carotene, vitamins A and C levels

Serum levels of β-carotene, vitamins A and C significantly decreased in DC group and increased (p<0.05) in the combined extracts treated group (DₓGLOG) when compared to normal control (table 3). These levels also increased significantly in extracts and insulin (Dᵢ) treated groups when compared to DC. The single extract treated groups displayed significant decrease (p<0.05) in the levels of these parameters as compared to combined extract treated groups.

Streptozotocin used in the induction of diabetes is a known pathogenic factor of free radicals generation or increased lipid peroxidation (Tatsuki et al., 1997; Szkudelski, 2001; Goycheva et al., 2006) and the resultant radicals do induce the antioxidant defense enzymes particularly in the liver, the tissue with the most abundant antioxidant defense enzymes (Tatsuki et al., 1997), causing an initial increase. But if this process continues and become chronic, the enzyme systems may become overwhelmed, and the induction system also exhausted or worn out hence leading to usually observed decrease in these antioxidant enzymes. The decrease is in line with the report of Ugochukwu et al. (2003) who suggested it to be as a result of the radicals inactivating the enzymes or glycation of the enzyme protein themselves. Other researchers also reported a
Table 2. Some oxidative stress indices in liver of treated and untreated diabetic rats

<table>
<thead>
<tr>
<th>Group/treatment</th>
<th>MDA (µmol/g protein)</th>
<th>SOD (U/g protein)</th>
<th>GPx (U/g protein)</th>
<th>CAT (U/g protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DC</td>
<td>30.25 ±0.04*</td>
<td>9.52 ±0.03*</td>
<td>8.40 ±0.03*</td>
<td>40.17 ±0.06*</td>
</tr>
<tr>
<td>GL</td>
<td>20.03 ±0.01,a,b,c</td>
<td>15.10 ±0.01*a,b,c</td>
<td>12.59 ±0.04*a,b,c</td>
<td>40.49 ±0.03*a,b,c</td>
</tr>
<tr>
<td>DG</td>
<td>19.72 ±0.01*a,b,c</td>
<td>15.20 ±0.05*a,b,c</td>
<td>12.65 ±0.01*a,b,c</td>
<td>45.25 ±0.02*a,b,c</td>
</tr>
<tr>
<td>DLOG</td>
<td>16.50 ±0.02*a</td>
<td>17.25 ±0.02*a</td>
<td>14.00 ±0.01*a</td>
<td>47.67 ±0.01*a</td>
</tr>
<tr>
<td>D1</td>
<td>15.50 ±0.05*a,b</td>
<td>19.70 ±0.02*a,b</td>
<td>16.20 ±0.02*a,b</td>
<td>50.24 ±0.01*a,b</td>
</tr>
<tr>
<td>NC</td>
<td>15.72 ±0.01</td>
<td>19.70 ±0.01</td>
<td>16.20 ±0.01</td>
<td>50.24 ±0.01</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM, n = 6.  
*p<0.05 vs DC; a = p<0.05 vs DC; b = p<0.05 vs DGL; c = p<0.05 vs D1

DC= Diabetic control;  
DGL= Diabetic treated with Gongronemalatifolium extract  
DLOG=Diabetic treated with Ocimumgratissimum extract  
D1=Diabetic treated with combined extracts of Gongronemalatifolium and Ocimum gratissimum  
NC= Normal control

Table 3. Serum β-carotene and vitamins A and C levels of treated and untreated diabetic rats

<table>
<thead>
<tr>
<th>Group/treatment</th>
<th>Vitamin A (µmol/l)</th>
<th>Vitamin C (mg/100ml)</th>
<th>β-carotene (µg/100ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DC</td>
<td>0.50 ±0.11*</td>
<td>0.33 ±0.01*</td>
<td>12.00 ±0.10*</td>
</tr>
<tr>
<td>GL</td>
<td>1.30 ±0.10*a,b,c</td>
<td>0.50 ±0.06*a,b,c</td>
<td>16.00 ±1.22*a,b,c</td>
</tr>
<tr>
<td>DG</td>
<td>1.31 ±0.09*a,b,c</td>
<td>0.52 ±0.02*a,b,c</td>
<td>16.06 ±0.59*a,b,c</td>
</tr>
<tr>
<td>DLOG</td>
<td>2.90 ±0.20*a</td>
<td>0.99 ±0.08*a</td>
<td>21.30 ±0.20*a</td>
</tr>
<tr>
<td>D1</td>
<td>1.50 ±0.05*a,b</td>
<td>0.58 ±0.01*a,b</td>
<td>16.60 ±0.02*a,b</td>
</tr>
<tr>
<td>NC</td>
<td>1.80 ±0.10</td>
<td>0.62 ±0.05</td>
<td>18.10 ±0.50</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM, n = 6.  
*p<0.05 vs NC; a = p<0.05 vs DC; b = p<0.05 vs DLOG; c = p<0.05 vs D1

DC= Diabetic control;  
DGL= Diabetic treated with Gongronemalatifolium extract  
DLOG= Diabetic treated with Ocimumgratissimum extract;  
D1= Diabetic treated with combined extracts of Gongronemalatifolium and Ocimum gratissimum  
NC= Normal control

decrease in the activity of these antioxidant enzymes in the liver of diabetic rats (Anuradha and Selvam, 1993; Stanelly et al., 2001). In this study, the significant increase in the level of MDA (an index of lipid peroxidation) and decrease in the activities of SOD, GPx and CAT in diabetic control group as compared to
normal control and extract treated groups might be linked to the production of ROS by STZ-induced hyperglycemia in rats. Ozmen et al. (2000) indicated in their report, an inverse relationship between ROS concentration and SOD, CAT and GPx activities in liver, heart and kidneys of diabetic animals. Lipid peroxidation is one of the characteristic features of chronic diabetes. The increased free radicals produced may react with polyunsaturated fatty acids (PUFA) in cell membranes leading to lipid peroxidation. The mechanism is that hydroxyl radical (OH) attacks polyunsaturated fatty acid (PUFA), forming a carbon-centred lipid radical. The radical rearrange to form a conjugate dienyl radical. This radical reacts with ambient oxygen (O₂), forming a hydroperoxyl radical, which then abstracts hydrogen from a neighboring lipid, forming lipid peroxide and starting a chain reaction. This reaction continues until the supply of PUFA is exhausted, unless a termination reaction occurs. GL and OG extracts (singly and in combination) may suppress lipid peroxidation through different chemical mechanisms, including free radical quenching, electron transfer, radical addition, or radical recombination (Liangli Yu et al., 2002). In this study insulin was more effective in reducing the MDA concentration than the extract while the extract was more effective in increasing the activity of antioxidant enzymes when compared to insulin group. This might be because the standard drug promoted the uptake of glucose into cells and therefore prevented hyperglycemic induced membrane peroxidation.

The significant (p<0.05) decrease in diabetic control rats (as compared to normal control) and increase in treatment groups (as compared to diabetic control) of serum levels of β-carotene, vitamins A and C could be linked to increased production of ROS and to the ability of the extracts to mitigate ROS generated by STZ diabetes respectively. Beta-carotene is the precursor of vitamin A, and its ability to act as an antioxidant is due to the stabilization of organic peroxide free radicals within its conjugated alkyl structure. Since β-carotene occurs at low oxygen concentration, it compliments antioxidant properties of vitamin E which is effective at higher oxygen concentrations. Vitamin C is known to act as an effective antioxidant on its own and it also shows excellent synergistic activity with vitamin E in the inhibition of oxygen radical-induced lipid peroxidation in vitro (Stocker, et al., 1986). Vitamin C within the body is maintained in the reduced form by shuttling the dehydroascorbate across the erythrocyte membrane for reconversion to ascorbate (Orroinger and Roear, 1979). Vitamin C exists as the enolate anion at physiological pH which spontaneously reduces superoxide, organic (Rt) and vitamin E radicals, forming dehydroascorboryl radical (AS). This radical undergoes a second reduction reaction to form dehydroascorbate which is recycled to ascorbate by dehydroascorbate reductase, a GSH-dependent enzyme present in all cells. Plants often contain substantial amounts of antioxidants including flavonoids, polyphenols, minerals (Se, Cu, Zn and Cr) carotenoids, β-carotene and vitamins such as tocopherol (vitamin E) and ascorbic acid (vitamin C), (Larson, 1988; Battel et al., 1999; Gorman, 1992). Vitamin E is the major chain terminating antioxidant in membranes; it reduces both conjugated dienyl and hydroperoxyl radicals, quenching the chain or cycle or lipid peroxidation reactions. This might have contributed to the enhanced activity of antioxidant enzymes displayed by the extract treated rats and reduced concentration of MDA, an index of lipid peroxidation.

CONCLUSION

The extracts of GL and OG showed a significant synergistic effect on STZ-induced diabetic rats. This implies that combined extract treatment may be more beneficial and useful relative to individual extracts in diabetes management as antioxidant defense system. Further studies are warranted to determine the exact components in GL and OG responsible for the observed synergistic effect and such components may be candidates for use as prophylactic agents against free radicals generated by STZ diabetes.

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