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Influence of Hydrocarbons on Plant Cell Ultrastructure and Main Metabolic Enzymes

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***Abstract*—**Influence of octane and benzene on plant cell ultrastructure and enzymes of basic metabolism, such as nitrogen assimilation and energy generation have been studied. Different plants: perennial ryegrass (*Lolium perenne*) and alfalfa (*Medicago sativa*); crops- maize (*Zea mays* L.) and bean (*Phaseolus vulgaris*); shrubs – privet (*Ligustrum sempervirens*) and trifoliate orange (*Poncirus trifoliate*); trees - poplar (*Populus deltoides*) and white mulberry (*Morus alba* L.*)* were exposed to hydrocarbons of different concentrations (1, 10 and 100 mM). Destructive changes in bean and maize leaves cells ultrastructure under the influence of benzene vapour were revealed at the level of photosynthetic and energy generation subcellular organells. Different deviations at the level of subcellular organelles structure and distribution were observed in alfalfa and ryegrass root cells under the influence of benzene and octane, absorbed through roots. The level of destructive changes is concentration dependent. Benzene at low 1 and 10 mM concentration caused the increase in glutamate dehydrogenase (GDH) activity in maize roots and leaves and in poplar and mulberry shoots, though to higher extent in case of lower, 1mM concentration. The induction was more intensive in plant roots. The highest tested 100mM concentration of benzene was inhibitory to the enzyme in all plants. Octane caused induction of GDH in all grassy plants at all tested concentrations; however the rate of induction decreased parallel to increase of the hydrocarbon concentration. Octane at concentration 1 mM caused induction of GDH in privet, trifoliate and white mulberry shoots. The highest, 100mM octane was characterized by inhibitory effect to GDH activity in all plants. Octane had inductive effect on malate dehydrogenase in almost all plants and tested concentrations, indicating the intensification of Trycarboxylic Acid Cycle.

The data could be suggested for elaboration of criteria for plant selection for phytoremediation of oil hydrocarbons contaminated soils.

***Keywords*—**Higher plants, hydrocarbons, cell ultrastructure, glutamate and malate dehydrogenases.

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I. Introduction

IN response to increased global pollution, progressively more attention is now being paid to the effective use of natural detoxifying agents. The understanding of the oil biodegradation process is becoming increasingly important. Foremost amongst these are microorganisms, which with their high transformation and degradation capabilities are successfully being used for the purification of sewage and soil [1].

Plants are also capable of absorbing and metabolizing many different types or organic pollutants, including hydrocarbons and are successfully applied in phytoremediation [2-7]. The pathways of absorption and transformation of different aliphatic, aromatic and polycyclic aromatic hydrocarbons in plants have been studied at Durmishidze Institute of Biochemistry and Biotechnology for more than 3 decades. Using ^{14}C-labelled aromatic hydrocarbons, such as benzene, nitrobenzene, aniline, benzoic acid, benzidine, 1,2-benzanthracene and 3,4-benzpyrene, was demonstrated that plants, grown under aseptic conditions are able to cleave aromatic ring [8-10].

Organic pollutants penetrated into plant cells cause significant changes across the whole range of intracellular metabolic processes. This is firstly manifested in the activation of inductive processes directed to the synthesis of enzymes participating in xenobiotics detoxification. Despite the fact that collateral biochemical processes accompanying detoxification process in plants are not well investigated, there are in the literature quite a few examples indicating that the activities of the enzymes participating in different regular cellular processes are also influenced by xenobiotics that have penetrated into the cell [11-13].

The aim of the paper is to study the influence of hydrocarbons on different plants cell structure and basic metabolic enzymes. Revelation of plants response to hydrocarbons, expressed in cell structure-function deviations, characteristic to each species, allows evaluating plants resistance to contaminated environment and prospects for their application in phytoremediation technologies.

II. Materials and Methods

A. Plants

Cops: monocotyledonous - maize (*Zea mays*) and dicotyledonous - bean *(Phaseolus vulgaris*); perennial herbs – ryegrass (*Lolium perenne*), and alfalfa (*Medicago sativa*);

evergreen shrubs – privet (*Ligustrum sempervirens*) and semi-evergreen - trifoliate orange (*Poncirus trifoliate*); trees - poplar (*Populus deltoides*) and white mulberry (*Morus alba* L.) were objects of the study.

B. Effect of Hydrocarbons

Young seedlings of plants and young shoots of shrubs and trees were exposed to benzene and octane mediums with hydrocarbons concentration – 1, 10 and 100 mM. Exposure time 5 days.

C. Electron Microscopy

Plant material was excised and 1mm^3 samples were fixed in a 2.5% solution of glutaraldehyde with postfixation in 1% osmium tetroxide. After dehydration in graded series of ethanol solutions, the samples were embedded in Epon-Araldite resin(1.5:1.0) and poured into gelatin capsules. Thin serial sections were made using Reichert and LKB III (Sweden) ultramicrotomes. Ultrathin sections were stained with uranyl acetate and examined in a Tesla BS 500 electron microscope [14].

D. Enzymes Assay

Plants were homogenized in 0.05 M phosphate buffer pH 7.5. The homogenate was centrifuged at 1000xg during 30 minutes. In resulted supernatants activities of enzymes – glutamate dehydrogenase (GDH), malate dehydrogenase (MDH) and glutamine synthetase (GS), as well as protein content were determined.

The GDH (EC 1.4.1.2) and MDH (EC1.1.1.37) activities were determined by a spectophotometric method according to the rate of oxidation of NADH at 340 nm [15, 16]. As a unit of enzymes activities the amount of enzyme that induced oxidation of 1 μmol of the NADH in min at 20^{0}C were taken. The reaction mixture for determining the amination activity of glutamate dehydrogenase contained: 0.1 ml of the solution to be investigated (40-60μg of protein), 10 mM of 2-oxoglutarate, 0.13 mM of NADH, and 100mM of NH_4Cl in a 0,05 M Tris-HCl buffer in a final volume of 3 ml (pH 7.8). Variants without ammonia, respectively, were served as the control.

The reaction mixture for determining the activity of MDH in reaction contained 0.02 ml of the solution to be investigated (10 μg of protein), 1.9 mM oxaloacetate, and 0.13 mM NADH in a 0,05 M Tris-HCl buffer in a final volume of 3 ml, pH of the mixture 7.8. Variants without oxaloacetate served as control.

The Glutamine synthetase (GS) activity (EC 6.3.1.2) was determined by a colorimetric method according to the amount of γ-glutamylhydroxamic acid (γ-GHA) formed in the transferase reaction [16]. The amount of the enzyme catalyzing the formation of 1 μmol of γ-GHA in 1 min at 37^{O}C was taken as the Glutamine synthetase activity unit.

Protein content was determined according to Bradford [17]. The specific activity of the enzymes was calculated as the number of units per 1 mg of protein in the solution under investigation.

III. Results and Discussion

A. Effect of Hydrocarbons on Plant Cell Ultrastructure

At low concentration, benzene vapour (0.04 mM) causes only insignificant changes on the cell ultrastructure of leaves of 7 day old bean seedlings, expressed in brightened fragments in the chloroplast matrix (Fig. 1. A). Increasing the benzene concentration fivefold (to 0.2 mM) leads to a destructive effect on chloroplasts, expressed in disorientation of lamellae and thylakoids (Fig. 1. B). Complete cell destruction occurs at 0.4 mM benzene (Fig. 1. C and D): in chloroplasts the internal membrane system is damaged and the matrix becomes electron dense, the cell wall is thickened, myelin and osmiophilic inclusions are observed in the periplasm and in vacuoles, and the mitochondria become electron dense.

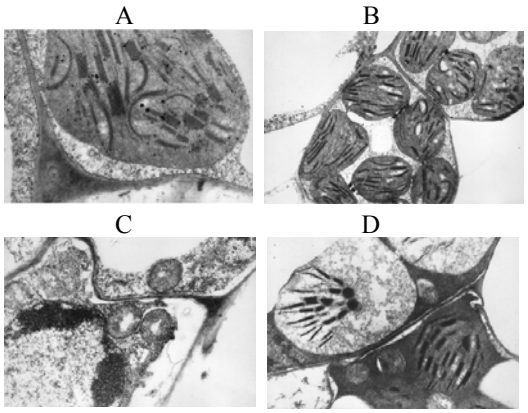

Fig. 1 Changes in bean leaves cells ultrastructure under the action of different concentrations of benzene vapour: A – 0.04 mM; B – 0.2 mM; C and D – 0.4 mM. (A - × 8 000; B, C and D - × 30 000)

Benzene vapour is less toxic to maize. At comparatively high, 0.5 mM concentration significant changes in chloroplasts ultrastructure was observed in cells of leaves of 7 day old seedlings. In particular, chloroplasts are extremely swollen (Fig. 2.) and invaginated; matrix of chloroplast is electron dense with a large quantity of lipid drops and vacuole like structures. The increase in number of contacts of swollen mitochondria with chloroplasts is observed, cytoplasm is strongly destructed, and cell wall is electron dense.

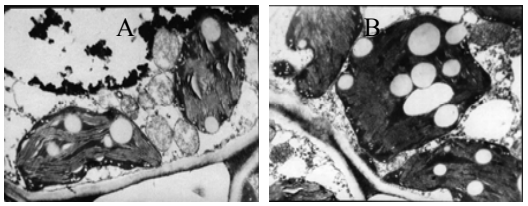

Fig. 2 Changes in maize leaf cells ultrastructure under the action of benzene vapour (0.5 mM by volume). × 10 000

The results indicate on different response of plants to benzene vapor, and maize appears to be resistant. Many woody plants have been reported to be highly resistant to benzene as it did not cause noticeable deviations in photosynthetic apparatus [18].

Destructive changes in chloroplasts structure was revealed by us earlier in plant leaves, exposed to gaseous alkanes in hermetic chambers: ethane, mixture of methane, ethane, propane and butane [19]. Alkenes, such as propylene and butylene were also shown to exhibit toxicity to photosynthetic apparatus of pea seedlings [18].

Later, the influence of benzene solution, absorbed through roots on ultrastructure of plants, as well as of octane has been studied.

Under the influence of benzene the root cells of ryegrass are completely destructed (Fig. 3, A, B, C). On the contrary, the structure of alfalfa root cells is not been infringed (Fig. 4, A, B). In cells of both plants great number of large size vacuoles with osmiophilic inclusions (Fig. 5B, 6A) and electrically dense cell walls and plastids are observed.

In the cell walls of ryegrass great number of liposomes are observed that indicates on active process of excretion (Fig. 3, A and C). In cells of alfalfa multiple contacts among mitochondria, plasmalemma, plastids and membranes of endoplasmic reticulum are seen (Fig. 4, B).

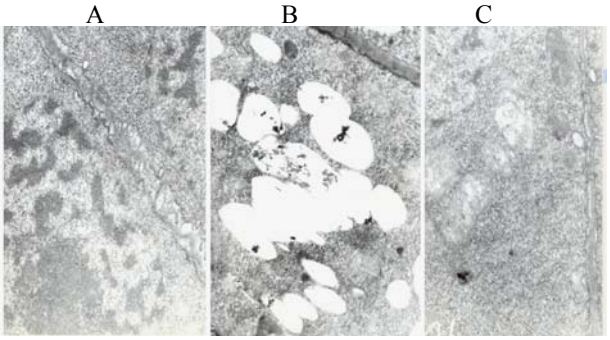

Fig. 3 Changes in ultrastructure of ryegrass root cortical cells under the influence of 0.1 mM benzene solution. A. x 28 000; B. x 20 000; C. x 36 000

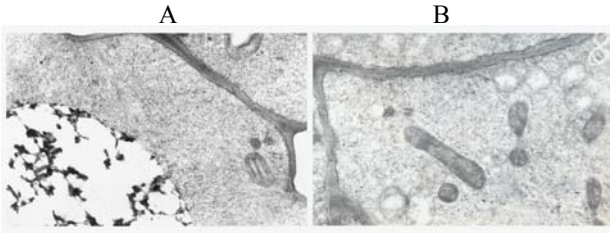

Fig. 4 Changes in ultrastructure of alfalfa root cortical cells under the influence of 0.1 mM benzene solution. . A. x 20 000; B. x 28 000

Under the influence of octane the root cells of ryegrass are completely destructed (Fig. 5, A). On the contrary, the main structure of alfalfa root cells is not infringed. The entirety of organelles is maintained, but some alterations in texture are observed. The number of mitochondria is increased; the cristae are swollen (Fig. 5, B and C). The plastids are bow-shaped with developed membrane structures (Fig. 5, B). The vacuoles are large and coupled with each other. They contain osmiophilic inclusions (Fig. 5, C and D). Ca^{2+}-binding sites, indicating on infringement of homeostasis are formed on tonoplasts. The cell wall is electrically dense (Fig. 5, B and C); the plasmalemma is invaginated, that points to the beginning of excretion of different compounds from vacuoles outside the cell. Frequent contacts among mitochondria of plasmalemma, plastids and membranes of endoplasmic reticulum are observed (Fig. 5, B).

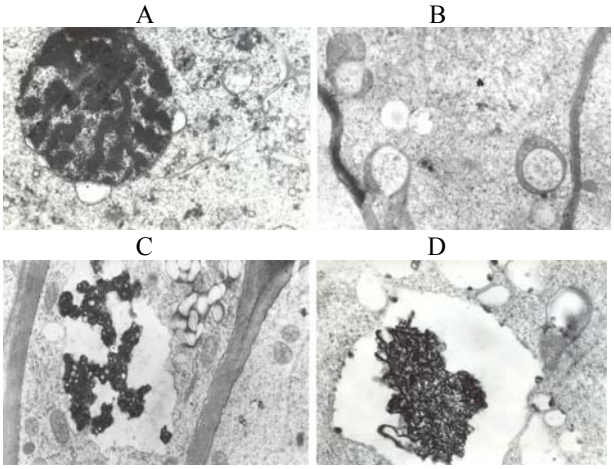

Fig. 5 Changes in ultrastructure of ryegrass (A; × 20 000) and alfalfa (B, C, and D; × 28 000) root cells under the influence of 0.1 mM octane

Both hydrocarbons, absorbed through roots are toxic to plants to different extent; the level of destructive changes is concentration dependent. Changes in root cells ultrastructure under the influence of different aromatic, including polycyclic aromatic hydrocarbons have been demonstrated earlier in annual plant seedlings [14, 20, 21].

B. Effect of Benzene and Octane on Enzymes of Nitrogen and Energy Exchange in Plants

Effect of benzene and octane was studied on main enzymes of energy and nitrogen metabolism, such as GDH, GS and MDH in leaves and roots of different plants.

GDH catalyzing reversible reaction – oxidative deamination of L-glutamate and reductive amination of 2-oxoglutarate and thus connecting nitrogen metabolism with the tri-carboxylic acid cycle, occupies the central position in cell energetics. Deamination, catalyzed by this enzyme is extremely important initial step in amino acid catabolism, which is intensified in cells under carbon deficiency. GS plays an important role in ammonia primary assimilation. The product of its activity, glutamine is donor of amino groups for biosynthesis of other amino acids, proteins, etc. GDH and GS play key roles in maintaining the balance of carbon and nitrogen in plant cell [22]. MDH, participating in the Tricarboxylic Acid Cycle (TCA) is involved in the processes of respiration and energy exchange. Study of the changes in the activities of these enzymes is extremely important for the understanding of plant resistance to different stresses, including contamination stress.

Results on influence of benzene on plant metabolic enzymes are given in Tables I-III.

As is seen, benzene lower concentrations (1 and 10 mM) cause significant 2-3 fold stimulation of GDH and MDH activities in the roots of ryegrass and maize seedlings (Table

1). In plant leaves the same induction effect is observed in case of ryegrass at benzene higher concentrations (10 and 100 mM) and in case of maize at benzene lowest concentration (1 mM).

Activity of GS in plant seedlings changes slightly.

TABLE I
CHANGES IN SPECIFIC ACTIVITIES OF GDH, GS AND MDH IN THE LEAVES AND ROOTS OF MAIZE AND RYEGRASS SEEDLINGS AFTER 5 DAYS EXPOSURE ON 1, 10 AND 100 MM BENZENE SOLUTIONS

Plant	Plant Organ	Benzene concentration, mM	GDH, μmol NADH/min per mg protein	GS, μmol γ–GHA/min per mg protein	MDH, μmol NADH/min per mg protein
Maize	Leaves	0 (Control)	0.024	2.38	0.13
		1	0.118	2.34	0.40
		10	0.078	1.79	0.23
		100	0.021	1.93	0.25
	Roots	0 (Control)	0.031	2.60	0.063
		1	0.106	1.65	0.139
		10	0.089	2.07	0.302
		100	0.068	1.52	0.535
Ryegrass	Leaves	0 (Control)	0.0090	0.258	0.455
		1	0.0063	0.211	0.692
		10	0.0142	0.176	0.395
		100	0.0136	0.289	1.228
	Roots	0 (Control)	0.0222	1.92	0.089
		1	0.0574	1.81	0.188
		10	0.0656	2.41	0.167
		100	0.0229	1.74	0.189

TABLE II
CHANGES IN SPECIFIC ACTIVITIES OF GDH, GS AND MDH IN PRIVET AND TRIFOLIAT ORANGE LEAVES AFTER 5 DAYS EXPOSURE OF SHOOTS ON 1, 10 AND 100 MM BENZENE SOLUTIONS

Plant	Benzene concentration, mM	GDH μmol NADH/ min per mg protein	GS, μmol γ–GHA /min per mg protein	MDH, μmol NADH/min per mg protein
European privet	0 (Control)	0.13	6.82	0.12
	1	0.18	4.29	0.19
	10	0.14	5.58	0.14
	100	0.05	5.83	0.13
Trifoliate orange	0 (Control)	0.16	1.16	0.28
	1	0.23	1.62	0.80
	10	0.15	0.76	0.23
	100	0.02	1.39	0.78

Benzene at concentration 1mM causes stimulation of glutamate and malate dehydrogenases activities in the leaves of both studied shrubs. Benzene higher concentrations (10 and 100 mM) cause inhibition of glutamate dehydrogenase and simultaneous increase in malate dehydrogenase activities, especially strong – by 180 % in trifoliate orange leaves (Table II).

Stimulation of glutamine synthetase activity is revealed only in trifoliate orange leaves at benzene lowest concentration (1mM) by 40 %.

TABLE III
CHANGES IN SPECIFIC ACTIVITIES OF GDH, GS AND MDH IN POPLAR AND WHITE MULBERRY LEAVES AFTER 5 DAYS EXPOSURE ON 1, 10 AND 100 MM BENZENE SOLUTIONS

Plant	Benzene concentration, mM	GDH, μmol NADH/min per mg protein	GS, μmol γ–GHA /min per mg protein	MDH, μmol NADH/min per mg protein
Poplar	0 (Control)	0.316	6.53	1.64
	1	0.236	3.89	1.24
	10	0.113	3.83	0.51
	100	0.057	9.68	0.63
White mulberry	0 (Control)	0.026	2.36	0.107
	1	0.059	2.32	0.091
	10	0.052	1.48	0.120
	100	0.031	1.95	0.100

Stimulation of GDH activity is observed in white mulberry leaves exposed to benzene, though, more significant at its lowest concentration. Contradictory, in leaves of poplar GDH activity inhibits parallel to the increase of benzene concentration (Table III).

Marked increase in GS activity is observed in the leaves of poplar only at benzene highest (100 mM) concentration.

Benzene in all tested concentrations causes inhibition of MDH activity in poplar leaves and does not affect it in white mulberry.

Effect of octane on GDH, GS and MDH in the leaves and roots of different plants are presented in Tables IV-V.

Octane at lower concentrations (1 and 10 mM) causes significant stimulation of GDH and GS activities in the leaves and roots of ryegrass and maize seedlings. At octane highest concentration stimulation of GDH activity in alfalfa and maize seedlings leaves, respectively by 460 and 700 % and simultaneous inhibition of the enzymes in plant roots, respectively by 96 and 50 % is observed. Stimulation of MDH activity is marked only in alfalfa leaves (Table IV).

Activity of glutamine synthetase increases only at octane highest concentration (100 mM) in ryegrass and maize leaves (respectively by 246 and 108 %) and in roots of alfalfa (by 302 %).

TABLE IV
CHANGES IN SPECIFIC ACTIVITIES OF GDH, GS AND MDH IN RYEGRASS, ALFALFA AND MAIZE LEAVES AND ROOTS AFTER 5 DAYS EXPOSURE ON 1, 10 AND 100 MM OCTANE SOLUTIONS

Plant	Plant organ	Octane concentration, mM	GDH, µmol NADH/ min per mg protein	GS, µmol× GHA* / min per mg protein	MDH, µmol NADH/ min per mg protein
Ryegrass	Leaves	0 (Control)	0.017	6.04	2.14
		1	0.093	5.49	1.79
		10	0.035	4.15	1.39
		100	0.023	4.13	0.92
	Roots	0 (Control)	0.115	2.07	1.59
		1	0.201	2.94	2.30
		10	0.150	2.47	1.95
		100	0.121	8.31	1.92
Alfalfa	Leaves	0 (Control)	0.110	0.28	0.32
		1	0.072	0.25	0.37
		10	0.133	0.33	0.54
		100	0.613	0.97	0.09
	Roots	0 (Control)	0.570	1.74	2.70
		1	0.191	0.66	0.89
		10	0.121	0.29	0.41
		100	0.021	2.11	1.58
Maize	Leaves	0 (Control)	0.017	3.44	1.92
		1	0.035	3.05	1.59
		10	0.031	3.18	1.80
		100	0.138	7.14	2.10
	Roots	0 (Control)	0.148	5.96	2.40
		1	0.400	8.89	5.56
		10	0.265	4.46	2.16
		100	0.072	0.15	1.19

TABLE V
CHANGES IN SPECIFIC ACTIVITIES OF GDH, GS AND MDH PRIVET, TRIFOLIAT ORANGE AND WHITE MULBERRY SHOOTS LEAVES AFTER 5 DAYS EXPOSURE ON 1, 10 AND 100 MM OCTANE SOLUTIONS

Plant	Octane concentration, mM	GDH, µmol NADH/ min per mg protein	GS, µmol× GHA/ min per mg protein	MDH, µmol NADH/ min per mg protein
Privet	0 (Control)	0.132	7.18	0.218
	1	0.179	5.74	0.080
	10	0.247	6.08	0.379
	100	0.058	2.95	0.042
Trifoliate orange	0 (Control)	0.066	3.00	0.491
	1	0.143	1.50	0.962
	10	0.041	1.52	0.922
	100	0.043	1.50	0.875
White mulberry	0 (Control)	0.033	4.35	1.30
	1	0.011	1.29	0.36
	10	0.017	0.99	0.33
	100	0.006	1.54	0.20

Under the influence of octane low concentration (1 mM) stimulation of GDH activity in the leaves of privet and trifoliate orange is revealed. At relatively higher concentration (10 mM), induction effect is more pronounced in privet leaves (by 87 %), while in trifoliate orange leaves inhibition of the enzyme activity (by 38 %) is observed (Table V).

Octane at highest concentration (100 mM) causes strong inhibition of GDH activity in leaves of all studied plants.

Octane causes stimulation of MDH activity in leaves of trifoliate orange; however the effect decreases parallel to the increase in hydrocarbon concentration.

Activity of glutamine synthetase is dicreased in all studied variants, indicating the inhibition of ammonia primary assimilation in plants exposed to octane [16].

Octane at all tested concentrations causes inhibition of all studied enzymes activities in white mulberry leaves (Table V), indicating high toxicity of this hydrocarbon to mulberry.

Some correlation is revealed between ultrastructural changes and activities of basic metabolism enzymes in plants exposed to hydrocarbons. For rexample, appearance of frequent contacts among mitochondria, plastids and membranes of endoplasmic reticulum parallel to activation of enzymes, involved in energy generation in alfalfa, under the influence of benzene and octane could be related to cell reorganization and mobilization of its resources for hydrocarbons detoxification.

IV. CONCLUSION

Measurable declinations in cell homeostasis were revealed at ultrastructural and basic metabolism enzyme's level in annual and perennial plants, exposed to benzene and octane.

Activation of GDH caused under the influence of hydrocarbons low concentrations indicates on participation of the enzymes in plant defense mechanisms, namely in intensification of amino acid catabolism, leading to energy generation which is in demand in cell under contamination stress.

The stimulation of MDH in plants exposed to octane increasing concentrations indicates to the intensification of TCA, probably for further oxidation of xenobiotic degradation products.

Assesment of declinations from normal range of metabolic processes under the influence of oil hydrocarbons allows elaborating plants selection criteria for their application in phytoremediation technologies.

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