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IN VIVO ACTIVATION OF GLUCURONYL TRANSFERASE IN RAT LIVER BY EUCALYPTOLE

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Abstract—Eucalyptole given either by aerosol or subcutaneously produced a marked increase in the activity of glucuronyltransferase, which slowly returned to normal. Repeated daily administrations (for up to 8 days) provoked the same response as a single one.

Studies of the ultrastructure of liver revealed no increase in smooth endoplasmic reticulum after eucalyptole aerosol. The different mechanism of phenobarbital-treated and eucalyptole-treated animals are discussed.

VESSEL[1] and Parke and Raham[2] reported an induction of drug-metabolizing enzymes in liver of mice and rats by terpinoids. Jori *et al.*[3] studied the effect of menthol, alpha- and beta-pinen, guaicol, eucalyptol and of the oil of Pinus pumilio on drug metabolism in rats. Only eucalyptole, administered either subcutaneously (s.c.) or by aerosol, increased the *in vitro* liver metabolism of aminopyrine, *p*-nitroanisol and anilin and the *in vivo* metabolism of pentobarbital.

Jori *et al.*[4] have shown, that eucalyptole given by aerosol to rats, increased the disappearance rate of amphetamine, zoxazolamine and pentobarbital from brain and plasma, but had no influence on that of phenylbutazone. An increased plasma disappearance was also shown to occur in man after 10 days treatment with eucalyptole aerosol.

The activity of glucuronyl transferase (GTA), assayed by glucuroconjugation of 4-methyl-umbelliferone, has been examined in crude-liver homogenates after administration of eucalyptole and various components of essential oils. The ultrastructure of rat liver after eucalyptole aerosol has also been studied.

EXPERIMENTAL

Male Sprague–Dawley rats weighing 150–180 g at the start of the experiment were used. For the eucalyptole inhalations three animals were placed in a cage. Undiluted eucalyptole was nebulized at a rate of 150 mg/min. The controls were treated in the same manner with aqua dest. in the first series, but in further trials the controls were untreated. In the first series of experiments (24 rats) the length of treatment was 5–8 days. For the other series (three times 35 rats) an experimental scheme was devised (see Table 2).

A geometrical series was chosen for the length of treatment (1, 2, 4 and 8 days), after which the animals were killed on different days, the end of treatment being called day 0 and the time elapsing between this zero day and the day of death also being divided into a geometrical series (0, 1, 2, 4, 8, 16 and 32 days). Eucalyptole and the

TABLE 1. EFFECT OF EUCALYPTOLE TREATMENT (AEROSOL) ON THE ACTIVITIES OF GTA-LEVELS IN LIVER

Treated animals			Control animals	
Expt. no.	Days of treatment	Enzyme activity in (nmoles 4-MU/mg protein/30 min)	Expt. no.	(nmoles 4-MU/mg protein/30 min)
1	5	392·3	13	352·2
2	5	386·6	14	347·2
3	6	395·8	15	349·9
4	6	394·3	16	322·9
5	7	415·2	17	315·0
6	7	413·1	18	343·5
7	8	443·8	19	325·0
8	8	427·2	20	342·7
9	5*	465·3	21	345·2
10	5*	422·4	22	366·6
11	5†	426·0	23	342·6
12	8‡	377·1	24	356·6
$\bar{X} \pm$ S.D.		413·27 ± 25·59	$\bar{X} \pm$ S.D.	342·45 ± 14·78

Twelve rats were treated with eucalyptole for varying times. On the first and second day of treatment the dosage was 150 mg/min for 5 min on the following days (3rd–8th) 150 mg/min for 10 min. Then the animals were killed and the enzyme level was assayed in crude liver homogenate.
$\bar{X} \pm$ S.D. = arithmetic mean ± standard deviation.
5* = treated for 5 days and killed 3 days after the last inhalation.
5† = treated for 5 days and killed 21 days after the last inhalation.
8‡ = treated for 8 days and killed 18 days after the last inhalation.

other components of essential oils, menthol, camphor, nutmegoil, thuja oil, turpentine oil and thymol, were administered subcutaneously (40 animals) for 4 days, eucalyptole in a dose of 500 and 1000 mg/kg, the other drugs in a dose of 500 mg/kg. The controls (five rats) received 500 mg/kg arachis oil, which was used for making dilutions.

Sodium-phenobarbital was administered intraperitoneally (i.p.) for 4 days to five animals (100 mg/kg) and saline i.p. to the controls (three rats).

The animals were killed by decapitation and the liver removed *in toto*. The GTA was assayed from a piece of liver, taken from the middle part of medium lobe.

Determination of GTA

An ultramicromethod, in principle the method of Perona *et al.*[5] was used, details of which have been described previously.[6] A mixture containing liver homogenate, UDPGA as glucuronate donor and 4-methylumbelliferone (4-MU) as glucuronate acceptor in tris buffer, pH 7·5, was incubated for 30 min at 37°.

The activity of GTA is given in nmoles 4-MU/mg protein/30 min. In all assays double samples from one liver homogenate were analysed.

Electron microscopical examination of rat liver

Small pieces of the liver were divided, one piece was used for the GTA-assay, the other piece was immersed in phosphate-buffered glutaraldehyde (6·25%, pH 7·4, 30 min). After prefixation the tissue blocks were washed twice in buffer solution and fixed for 1 hr in 1% OsO_4 (pH 7·4, 4°).

After washing in buffer, the tissue was dehydrated in acetone (25, 50, 70, 90% 30

TABLE 2. TIMETABLE FOR THE TREATMENT WITH EUCALYPTOLE AEROSOL 150 mg/min FOR 10 min

Days of treatment	Group 1 8 days treated	Group 2 4 days treated	Group 3 2 days treated	Group 4 1 day treated	Control untreated
—8	1 2 3 4 5 6 7				
—7	1 2 3 4 5 6 7				
—6	1 2 3 4 5 6 7				
—5	1 2 3 4 5 6 7				29 30 31 32 33 34 35
—4	1 2 3 4 5 6 7				29 30 31 32 33 34 35
—3	1 2 3 4 5 6 7	8 9 10 11 12 13 14			
—2	1 2 3 4 5 6 7	8 9 10 11 12 13 14			
—1	1 2 3 4 5 6 7	8 9 10 11 12 13 14	15 16 17 18 19 20 21		
	1 2 3 4 5 6 7	8 9 10 11 12 13 14	15 16 17 18 19 20 21	22 23 24 25 26 27 28	
Interval in days between end of treatment and day of killing					
0	1	8	15	22	29
1	2	9	16	23	30
2	3	10	17	24	31
4	4	11	18	25	32
8	5	12	19	26	33
16	6	13	20	27	34
32	7	14	21	28	35

Four groups of rats consisting of seven animals were subjected to eucalyptole aerosol for varying times as indicated in the table, seven rats served as controls. The animals were killed at different days after treatment as indicated in the second half of the table.

min and three times 100% 15 min). The samples were embedded in Durcupan ACM, polymerised at 60° for one week and cut with glass knives using the Reichert UM 2. The sections were stained with lead hydroxide.[7] Electron-micrographs were made on Gaevert Scientia 23 D 50 at two levels of magnification (2000 and 10,000) in a Siemens Elmiscop I electron microscope. Morphometric data[8] were sampled with a square grid laid over the negatives and observed at low magnification (3 and 5).

RESULTS

(a) *Biochemical results*

(1) Table 1 shows a significant increase of GTA in rat liver after eucalyptole inhalation for 5–8 days. In the animals which were killed 3–21 days rather than immediately after the end of treatment, an elevated enzyme level was also observed.

TABLE 3. EFFECT OF EUCALYPTOLE AEROSOL ON THE ENZYME LEVEL EXPRESSED AS nmoles 4-MU/mg PROTEIN/30 min

	Distance in days between the end of treatment and day of killing	Control Animals	Treatment with eucalyptole in days 1	2	4	8
Expt. No. I	0	335·2	337·4	369·8	384·1	416·1
	1	355·4	356·9	399·2	416·9	384·0
	2	318·0	433·0	441·0	425·2	416·9
	4	327·7	438·4	409·5	402·8	413·7
	8	290·8	424·5	435·8	372·9	363·3
	16	333·5	417·1	400·6	381·6	354·8
	32	301·9	347·1	405·5	322·6	383·9
Expt. No. II	0	313·1	352·1	378·4	386·0	385·4
	1	322·1	384·5	381·2	406·7	389·2
	2	289·9	377·5	403·8	407·5	417·8
	4	326·3	436·1	395·0	411·9	419·6
	8	337·5	398·4	388·5	403·4	396·1
	16	347·1	357·1	342·3	352·1	346·9
	32	337·4	342·1	341·5	307·1	352·2
Expt. No. III	0	347·0	383·5	404·6	410·0	449·1
	1	342·8	407·1	381·5	388·7	390·5
	2	332·9	422·5	397·5	420·1	441·5
	4	323·2	399·2	362·5	352·7	357·2
	8	291·3	374·3	381·5	321·6	311·0
	16	340·2	347·4	360·4	368·9	345·0
	32	320·0	313·4	302·1	316·9	327·1
Arithmetic mean out of Expt. No. I, II and III	0	331·8	357·7	384·3	393·4	416·9
	1	340·1	382·8	387·3	404·1	387·9
	2	313·6	411·0	414·1	417·6	425·4
	4	325·7	424·6	389·0	389·1	396·8
	8	306·5	399·1	401·9	366·0	356·8
	16	340·3	373·9	367·8	367·5	348·9
	32	319·8	334·2	349·7	315·5	354·4

The values in experiment I, II and III are arithmetic means of two independent measurements of the same liver homogenate. The GTA-activities in the treated animals are attained after different length of treatment and after a different interval between the end of treatment and the day of killing, as indicated in the Table 2.

(2) This led us to undertake a second series of experiments with eucalyptole aerosols (Table 2). Besides the control groups (0 days of treatment) of seven rats, four groups of seven rats were treated for different lengths of time (1–8 days) and killed 0–32 days after the last inhalation. Both the duration of treatment and the time elapsing before death were arranged in a geometrical series, each combination of values representing one rat. The experiments were repeated three times.

Table 3 shows the enzyme levels for each rat in this experiment. These are arithmetic means of two independent measurements on the same liver homogenate.

The data were evaluated by means of analysis of variance. Despite identical treatment the enzyme activities in the three groups of replication differed significantly at the 99·9 per cent level. The reason for this might be, that the three sets of experiments could not be carried out simultaneously. These differences, however, will not interfere with the following statistical calculations. The increase after treatment is significant at the 99·9 per cent level, also the difference between control and treated animals; but between groups of animals treated for different length of time no statistically significant difference could be observed. The differences in enzyme activity between groups of animals killed at different times after end of treatment are also significant but at the lower level of 95 per cent.

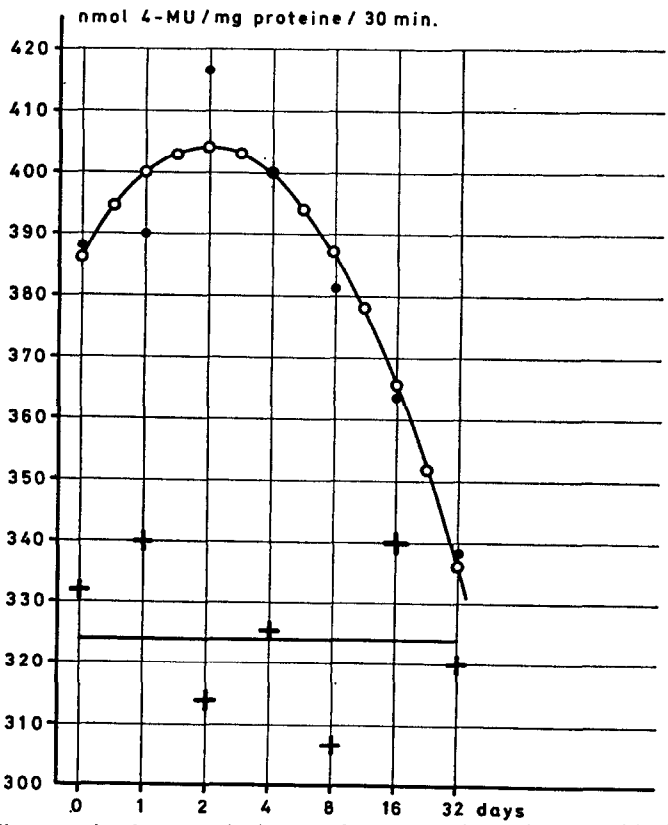

FIG. 1. Parabolic regression between the interval 0–32 days, plotted in a logarithmic scale and the enzyme level GTA in nmole 4 MU/mg protein/30 min of treated and control animals. ○ = Calculated values, ● = observed means of treated animals (12 rats with different length of treatment), + = observed values of controls (three rats).

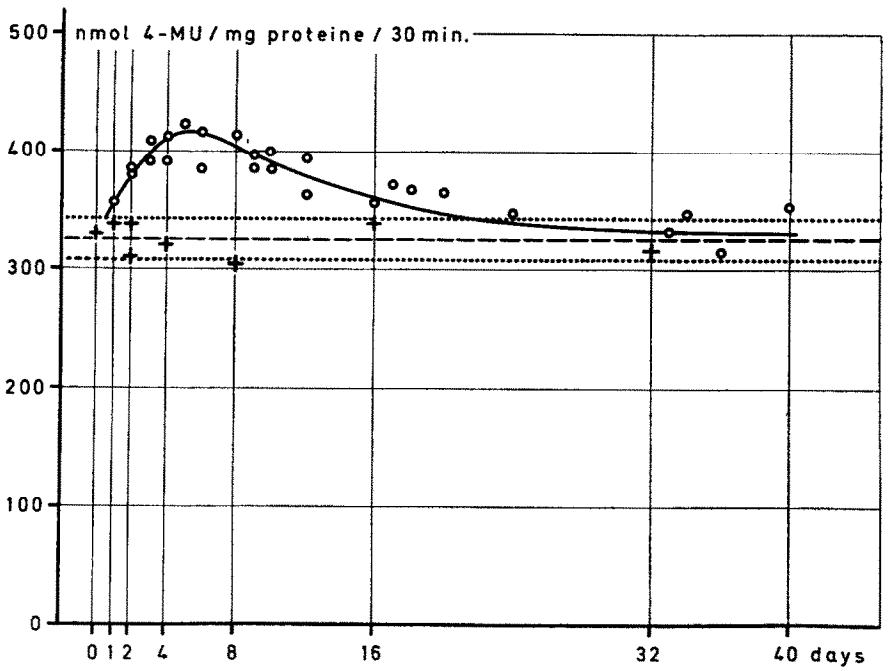

FIG. 2. Enzyme activities of treated and control animals plotted against the interval 0–40 days (between beginning of treatment and time of killing). The activity of GTA is expressed as nmoles 4-MU/mg protein/30 min. ○ = Means after eucalyptole aerosoltreatment (three rats), + = means of control animals (three rats), - - - - = arithmetic mean of values of control animals, ... = confidence limits at the 99·9% level, ——— = eye-fitted curve.

In Fig. 1 a parabolic regression is constructed by plotting on a logarithmic scale the interval between end of treatment and time of killing against linear enzyme activity. The parabolic regression was found by orthogonalic partition, which leads to a negative linear and a negative quadratic component both of which are statistically highly significant. The position of the maximum of the calculated curve is determined by the experimental values. For this procedure all data based on treatments of varying duration were combined.

In Fig. 2 enzyme activities are plotted against a non-logarithmic time scale, which in this case represents the interval between beginning of treatment and time of killing. This procedure is justified, because the statistical analysis did not demonstrate any influence of the length of treatment. The figure shows the rapid increase during the first 3 days after the beginning of treatment, a high and nearly constant value within the 3rd–10th day and a subsequent slow decrease. The eye-fitted curve approaches the normal range of enzyme activity on about the 24th day. The shape of the curve is independent of the length of treatment.

(3) Table 4 shows that subcutaneous administration of eucalyptole (500 and 1000 mg/kg) also activates GTA. The dependence upon dosage is significant with 95 per cent probability but more experiments must be performed.

No influence on enzyme activity was seen following treatment with menthol, thuja oil, nutmeg oil, turpentine oil and thymol. Animals treated with camphor s.c. showed

TABLE 4. EFFECT OF EUCALYPTOLE (500 AND 1000 mg/kg) S.C. AND OF OTHER COMPONENTS OF ESSENTIAL OILS, CAMPHOR, MENTHOL, NUTMEGOIL, TURPENTINE OIL, THUJA OIL AND THYMOL (500 mg/kg) S.C. FOR 4 days ON THE GTA-LEVEL

Drug	Application	Dosage (mg/kg)	Treatment (days)	Numbers of animals	Enzyme activity in nmoles 4-MU/mg protein/30 min Arithmetic mean	Minimum–Maximum
Eucalyptole	s.c.	500	4	5	408·09	392·05–422·65
Eucalyptole	s.c.	1000	4	5	432·38	405·20–445·15
Camphor	s.c.	500	4	5	365·50	351·20–385·50
Menthol	s.c.	500	4	5	332·90	307·00–352·05
Nutmeg oil	s.c.	500	4	5	346·30	319·70–380·50
Turpentineoil	s.c.	500	4	5	348·20	341·50–364·90
Thuja oil	s.c.	500	4	5	352·10	342·10–359·70
Thymol	s.c.	500	4	5	329·62	281·90–352·90
Arachis oil	s.c.	500	4	5	344·50	326·30–357·10
Na-phenobarbital	i.p.	100	4	5	429·50	407·10–454·60
Phys. NaCl	i.p.	100	4	3	345·20	331·60–356·90

The control animals were treated with arachis oil for 4 days. The second part of the table shows the effect of sodium phenobarbital (100 mg/kg) i.p. for 4 days on the enzyme activity; saline was administered to the controls i.p.

The arithmetic means and the ranges of enzyme activities in nmoles 4-MU/mg protein/30 min are given.

a statistically significant difference compared with the controls ($P > 0{\cdot}001$) but the effect was very low.

(4) Table 5 shows that the ratio of wet liver weight to total body weight of rats increases in phenobarbital-treated animals and in eucalyptole (s.c.) treated animals. The increase in this ratio shows nearly the same dependence on dosage as enzyme activity.

TABLE 5. EFFECT OF EUCALYPTOLE GIVEN SUBCUTANEOUSLY AND SODIUM PHENOBARBITAL INTRAPERITONEALLY (i.p.) ON THE RATIO OF WET LIVER WEIGHT TO TOTAL BODY WEIGHT IN % OF MALE SPRAGUE–DAWLEY RATS WEIGHING 201–266 g

Eucalyptole s.c. (500 mg/kg)	Wet liverweight/body weight (%)	Eucalyptole s.c. (1000 mg/kg)	Wet liverweight/body weight (%)
1	3·90	1	4·30
2	4·00	2	4·00
3	3·75	3	4·30
4	4·10	4	4·30
5	3·75	5	4·50
$\bar{X}$	3·90	$\bar{X}$	4·26
Na-phenobarbital (i.p. 100 mg/kg)		Controls saline (i.p. 100 mg/kg)	
1	4·46	1	3·27
2	4·84	2	3·12
3	4·49	3	3·31
4	4·20	4	3·00
$\bar{X}$	4·49	$\bar{X}$	3·17

The animals were treated with eucalyptole 500 and 1000 mg/kg for 4 days with sodium phenobarbital 100 mg/kg for 4 days. To the control animals saline was applicated i.p., $\bar{X} \pm$ arithmetic mean.

(b) *Results of electron microscopy*

Only the livers of the first series of experiments were fixed and embedded for electron microscopy. A survey shows, that on the ultrastructural level no obvious difference between controls and treated groups could be seen. Therefore morphometric measurements were only performed on the rats treated for 5 days and their controls (Table 6). In particular, mitochondrial volume was measured as percentage of cell volume (with a point lattice). From the mitochondria also the total inner surface area, that is including the cristae, were measured in relation to the unit volume of mitochondria and the unit volume of cytoplasma. The surface area of smooth and rough endoplasmic reticulum (e.r.) per unit volume cytoplasma were also measured. No significant increases or decreases of these compartments of the liver cell could be found.

DISCUSSION

Jori *et al.*[3,4] treated male Sprague–Dawley rats with different components of essential oils by aerosol inhalation or s.c. administration and determined the activation of drug metabolism *in vivo* as well as *in vitro* with a 9000 *g* supernatant of liver homogenate. Only eucalyptole increased the rate of disappearance of the different drugs

TABLE 6. QUANTITATIVE MEASUREMENTS ON ELECTRON MICROGRAPHS OF MITOCHONDRIA, SMOOTH AND ROUGH ENDOPLASMIC RETICULUM (e.r.) OF RAT LIVER CELLS

Cell compartment	Numbers of animals	After eucalyptole aerosol	Controls
Mitochondrial volume (%)	5	20·5 ± 4·2	19·8 ± 4·0
Inner mitochondrial membrane surface per area mitochondrial unit volume (μ^2/μ^3)	5	25·8 ± 4·0	24·9 ± 3·2
Inner mitochondrial membrane per cytoplasma unit volume (μ^2/μ^3)	5	5·29 ± 0·9	4·90 ± 0·8
Surface area of smooth e.r. per cytoplasma unit volume (μ^2/μ^3)	5	3·4 ± 1·2	3·3 ± 0·96
Surface area of rough e.r. per cytoplasma unit volume (μ^2/μ^3)	5	5·1 ± 1·1	4·98 ± 0·90

examined. This effect could be confirmed by us examining the activity of GTA, which is a key enzyme not only in drug metabolism but also in endogenous detoxification. The same authors administered the aerosols for 4 days and measured the effect on drug metabolism up to 72 hr. Instead of a single duration of treatment with some short intervals between the last treatment and examination of drug metabolism, we chose varying lengths of treatment and a longer period between the end of treatment and determination of enzyme activity. A logarithmic scale was used. In contrast to the relatively short time (36 hr) during which Jori *et al.*[3,4] found an elevated activity of drug metabolism, a highly increased activity of GTA was established even after a long period (up to 20 days). Furthermore it was shown that a single treatment of eucalyptole aerosol suffices to elevate the GTA in the same range as all other aerosol treatments for up to 8 days of daily applications.

Our trials with s.c. administrations were intended to show which other components of essential oils are able to stimulate the GTA. Only camphor produced a small response. The effects of subcutaneous administration of eucalyptole (500 and 1000 mg/kg) after 24 hr are slightly dependent on dosage, but from our experience with eucalyptole aerosol trials we conclude, that it would be necessary to observe the enzyme activity over a long period to decide, if the mechanism of aerosol and subcutaneous administrations is different. Considering the first values of aerosol treatments only, a well established dependence on dosage has been demonstrated. However, following the enzyme levels over a longer period it is obvious that, if there is any dosage dependence it is very small. Contrary to these findings Jori *et al.*[3,4] could not demonstrate any effect of eucalyptole treatment after 48 and 72 hr, but detected a response after 36 hr measured by sleeping time or by pentobarbital levels in the brain. In this respect the findings of these authors agree with the results after treatment with phenobarbital.[9] The mechanism of phenobarbital action is believed to resemble the mechanism of enzyme induction as described by Jacob and Monod.[10]

However, the mechanism of eucalyptole by aerosol treatment has to be quite different from the mechanism of phenobarbital in three ways.

Firstly, the change of enzyme activity with time resembles the change of drug

concentration in blood serum. Contrary to the rapid decrease after phenobarbital the effect of treatment of Eucalyptole disappears only gradually.

Secondly, after the end of treatment enzyme activity continues to increase.

Thirdly, induction of enzyme activity is caused by a single treatment of Eucalyptole, whereas several doses of phenobarbital are required.

Jori *et al.*[3,4] suggested that the effect of Eucalyptole is the same as phenobarbital treatment in induction of smooth e.r. After phenobarbital treatment Staubli *et al.*[11] could measure an increase in smooth e.r. We measured e.r. and mitochondrial configuration of liver cells in controls and treated rats, but we were unable to find any influence of Eucalyptole aerosol administrations. An increasing number of papers show clearly, that a good correlation exists between physiological data and morphometric measurements.[12–17] Besides the increased smooth e.r. after phenobarbital application also the cell volume, the relative ratio of liver weight to total body weight and the numbers of mitoses increases.[18,19] These investigations suggest, that the mechanism of the induction of phenobarbital is more complex than a single enzyme induction.

Also subcutaneous administration of Eucalyptole demonstrated the higher ratio between wet liver weight and total body weight, the cell volume or the mitoses in livers of eucalyptole-treated rats were not measured.

It would be desirable not only to determine the activity of GTA but also the amount of enzyme. It is clear, that many other mechanisms beside enzyme synthesis must be involved in producing the striking differences on the action of the two drugs.

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