Peroxidase-Activated Carcinogenic Azo Dye Sudan I (Solvent Yellow 14) Binds to Guanosine in Transfer Ribonucleic Acid

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Abstract. Peroxidase in the presence of hydrogen peroxide catalyzes *in vitro* the activation of the carcinogenic azo dye Sudan I (1-phenylazo-2-hydroxynaphthalen) to tRNA-, homopolyribonucleotide- and 5′-monophosphate nucleoside-bound products. tRNA, poly G and guanosine 5′-monophosphate modified by activated Sudan I become colored and have an absorption maximum of approx. 480 nm. Cochromatographic analysis of adducts obtained by a reaction with tRNA and guanosine 5′-monophosphate on a thin layer of cellulose showed that the major Sudan I-tRNA adduct was formed by a reaction of activated Sudan I with guanosine in tRNA. The radical mechanism of the binding of the Sudan I molecule, containing the whole azo aromatic system, to nucleic acids is discussed.

Key words: Carcinogen — Azo dye — Activation — Peroxidase — Binding to tRNA.

Introduction

Sudan I (1-phenylazo-2-hydroxynaphthalene, Solvent Yellow 14) has been studied as a model non-aminoazo dye. It has been used as a food colorant in several countries (IARC 1975), but was classified unsafe as a carcinogenic compound inducing tumors in the liver and/or the urinary bladder of rats, mice and rabbits (IARC 1975; NCI 1982; Garner et al. 1984; Westmoreland and Gatehouse 1991). Nevertheless, it is still widely used to color other materials (IARC 1975). Microsomal cytochromes P-450 are supposed to be responsible for the detoxication (the formation of C-hydroxyderivatives) as well as for the activation of Sudan I (the splitting of the molecule to yield the benzenediazonium ion (BDI), which binds

Dedicated to our teacher, Prof. S. Leblová, on the ocassion of her 70th birthday.

to nucleic acids) in the liver (Childs and Clayson 1966, Stiborova et al 1988a,b, 1990b, Stiborova et al 1995)

A limited role has, however, been suggested for cytochromes P-450 in the *in vivo* metabolic activation of Sudan I in the urinary bladder This organ has little or no detectable cytochrome P-450, but peroxidase is present at relatively high levels (Yamazoe et al 1985, 1988) As *in vitro* Sudan I is very effectively activated by peroxidase, forming covalent adducts with DNA (Stiborova et al 1988c, 1990b), transfer RNA (tRNA) (Stiborova et al 1990a) or proteins (Stiborova et al 1991), we suggested this enzyme to be potentially responsible for the initiation of chemical carcinogenesis in the urinary bladder (Stiborova et al 1988c, 1990a,b) tRNA is more susceptible than DNA to modification by peroxidase-activated Sudan I (Stiborova et al 1988c, 1990a,b) Moreover the physiological function of tRNA is changed due to modification by activated Sudan I Namely, tRNA modified by activated Sudan I exhibits a significantly increased acceptance for L-methionine (Stiborova et al 1990 a)

The present paper continues our previous study, and aims at identifying the target ribonucleotides in tRNA for covalent binding of Sudan I by comparing the properties of adducts obtained from tRNA and from the individual homopolyribonucleotides (poly X) or 5′ phospho-nucleotides [guanosine 5′-monophosphate (GMP), adenosine 5′-monophosphate (AMP), uridine 5′ monophosphate (UMP) and cytidine 5′-monophosphate (CMP)]

Abbreviations: AMP, adenosine 5′-monophosphate, BDI, benzenediazonium ion, CMP, cytidine 5′-monophosphate, GMP, guanosine 5′-monophosphate, NMR, nuclear magnetic resonance, poly X, homopolyribonucleotides, R_f, relative mobility, TLC, thin layer chromatography, tRNA, transfer RNA, UMP, uridine 5′-monophosphate, UV/vis spectroscopy, ultraviolet/visible spectroscopy

Materials and Methods

Chemicals and radiochemicals

Chemicals were from the following sources peroxidase (horseradish) from Boehringer, Mannheim FRG Sudan I from British Drug Houses, Poole, UK, GMP, CMP, AMP and UMP from Serva, Heidelberg, FRG, homopolymers (poly X) were from Pharmacia LKB, Uppsala, Sweden, and nuclease P1 from Sigma, St Louis, Mo, USA, rat liver tRNA was prepared according to Rogg et al (1969), all other chemicals were reagent grade or higher

^{14}C-labeled Sudan I (0 54 mCi/mmol) was synthesized from [U-^{14}C] aniline (2 5 mCi/mmol, Ammersham International plc, Amersham, UK) and non-labeled β-naphthol (Matrka and Pipalova 1982) and purified by column chromatography on basic alumina and by chromatography on thin layer of Silicagel G (Woelm) The labeled compound was stored in methanol at −17 °C

Incubations

tRNA was modified by peroxidase-activated Sudan I and isolated as described previously (Stiborová et al. 1990a). Briefly, 0.1–0.2 mmol/l [^{14}C]Sudan I was incubated in a final volume of 1.5 ml with 0.2 mg horseradish peroxidase, 1.5 mmol/l hydrogen peroxide and 1–2 mg of tRNA in 50 mmol/l Tris-HCl buffer (pH 8.4) at 37 °C, for 20 min. The mixtures were extracted twice with ethyl acetate. tRNA was isolated by phenol/chloroform extraction (2x) and precipitated by ethanol (Stiborová et al. 1990a). Precipitates of tRNA were washed with ethanol, ethanol/diethyl ether (1:1, v/v), diethyl ether (2x), and dried under a stream of nitrogen. tRNA was dissolved in one ml of distilled water and dialyzed against distilled water (for 24 hours). The ^{14}C-radioactivity of tRNA was determined in 0.1 ml aliquots by liquid scintillation counting (Packard Tri-Carb 2000 CA). The same experimental conditions were used to modify polyribonucleotides (poly X), except that poly G, poly A, poly U and poly C were used instead of tRNA. The tRNA and poly X content was quantified spectrophotometrically (Rogg et al. 1969).

The same experimental conditions were also used to modify 5′ monophosphate nucleosides, except that 6 mg of GMP, AMP, UMP or CMP was used instead of tRNA or poly X. The mixtures were extracted twice with ethyl acetate, and ^{14}C-radioactivity and absorbance at 480 nm were measured in aliquots of water phases.

^{14}C-radioactivity was measured using scintillation counting in an Instagel or Packard Ultra Gold XR liquid scintillator in a Packard Tri-Carb 2000 CA scintillation counter. Absorbance at 480 nm and/or UV/vis spectra were measured on a Perkin-Elmer Lambda 5 spectrophotometer.

Hydrolysis of tRNA modified by peroxidase-activated [^{14}C]Sudan I, and resolution of adducts

One mg of tRNA modified by peroxidase-activated [^{14}C]Sudan I (see above) was dissolved in 0.1 ml of distilled water and enzymatically hydrolyzed by nuclease P1. Aliquots of 0.1 ml of the mixture containing 5 μl H_2O, 9 μl 1 mol/l sodium acetate (pH 5.0), 32 μl 1 mol/l $ZnCl_2$ and 45 μl nuclease P1 (4 μg/μl) were added to 0.1 ml of samples containing tRNA, and incubated at 70 °C for 60 minutes (Fujimoto et al. 1974). After cooling to 4 °C, the digest was extracted twice with 200 μl of ethyl acetate, the ethyl acetate layers were collected and the residual water layers were dried in a stream of nitrogen to remove residual ethyl acetate. The samples were chromatographed on a column of Separon SGX C 18 (Presep) previously washed with the mixtures of methanol/NH_4OH (4 N) (1:1, v/v) and distilled water. The column was washed with 50 ml of distilled water. The tRNA digest was eluted from the column by 0.5 ml of isopropanol/NH_4OH (4 N) (1:1, v/v). The eluate was evaporated in a stream of nitrogen to dryness and the hydrolysate was dissolved in 20 μl of NH_4OH (14 N)/*n*-butanol/*n*-propanol/H_2O (35:35:25:10, v/v), applied on thin layer plates of cellulose (Merck, Darmstadt, FRG) and developed in the same solvent system (see above). The colored adduct zones and further zones detected as UV positive were scraped from the plates and the radioactivity was determined by scintillation counting. Alternatively, the radioactive adduct spots with relative mobilities (R_f) corresponding to that of the major GMP-Sudan I adduct (R_f = 0.71) were extracted with isopropanol/NH_4OH (4 N) (1:1, v/v), evaporated to dryness under vacuum, dissolved in distilled water, centrifuged, and the clear solutions were used for UV/vis spectroscopy.

The same experimental procedure (chromatography) was used for the purification of adducts formed from GMP and peroxidase-activated [^{14}C]Sudan I and for the isolation

of the major GMP-Sudan I adduct Excess GMP which was not modified by Sudan I was precipitated with five volumes of acetone, the precipitated GMP was centrifuged (1000×g, 10 minutes at 4 °C, Janetzki K-25, Germany) and the collected supernatant containing acetone was evaporated in a stream of nitrogen to a minimum volume

Results

Binding of [^{14}C]Sudan I to tRNA and homopolyribonucleotides

Peroxidase in the presence of H_2O_2 is effective in supporting the activation of Sudan I to products binding to tRNA and homopolyribonucleotides Polyribonucleotides were used instead of tRNA in parallel experiments for the identification of the target ribonucleotides for the peroxidase-mediated Sudan I binding As shown in Table 1, the extent of polyribonucleotide binding was highest to poly G and proceeded in the order poly G > poly A > poly U > poly C Only adducts formed from poly G and poly A were colored, having an absorption maximum at pH 7 0 at about 480 nm, similarly as Sudan I-tRNA adducts (Stiborova et al 1990a) Poly U and poly C adducts were colorless Figure 1 shows absorption spectra of adducts formed from peroxidase-activated Sudan I and poly G under neutral and alkaline conditions A shift of the absorption maximum of the poly G adduct (480 nm at pH 7 0) to 468 nm was observed upon increasing pH to 8 4 (Fig 1) These absorption maxima are also typical of the spectrum of Sudan I, tRNA-Sudan I and/or DNA Sudan I adducts (Stiborova et al 1988a, 1990a,b, 1994) This could mean that the entire peroxidase-activated Sudan I molecule gets bound to poly G (or tRNA or DNA) molecules, being responsible for the color of modified nucleic acids

Binding of [^{14}C]Sudan I to nucleotides

[^{14}C]Sudan I activated by the peroxidase system was let to react with major nucleotides of tRNA, namely, GMP, AMP, UMP and CMP To remove resid-

Table 1. Binding of peroxidase-activated [^{14}C]Sudan I to tRNA and poly X

Compound	Binding (nmol mg^{-1})
tRNA	14 80 ± 0 91
Poly G	10 40 ± 0 80
Poly A	4 00 ± 0 42
Poly U	2 90 ± 0 30
Poly C	1 90 ± 0 21

For experimental conditions see the text The average binding levels and standard deviations were obtained from triplicate determinations

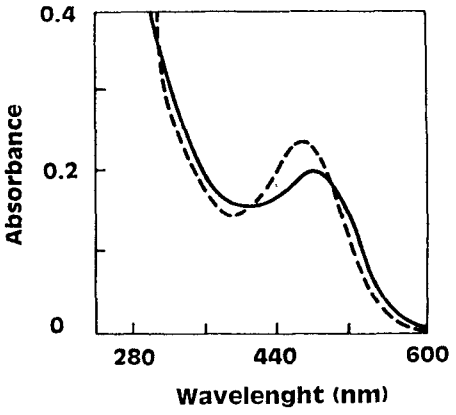

Figure 1. Absorption spectra of poly G (0.1 mg/ml) modified by Sudan I activated by the peroxidase system at pH 7.0 (———) and pH 8.4 (– – – –). The spectra were measured using a Perkin-Elmer Lambda 5 UV-visible spectrophotometer.

ual [^{14}C]Sudan I and its metabolites, the reaction mixtures were extracted twice with ethyl acetate. ^{14}C radioactivity of the peroxidase-mediated metabolites of [^{14}C]Sudan I bound to the respective nucleotides corresponding to the adducts was then measured in residual water phases. Moreover, as Sudan I and its metabolites absorbing at 480 nm (Stiborová et al. 1990a,b; 1994) were expected to be bound to nucleotides, we also determined the absorbance at 480 nm in the residual water phases. It should be mentioned that the quantitation of adducts formed from nucleotides and of activated [^{14}C]Sudan I as estimated by both above methods is not absolute. A low amount of highly water-soluble Sudan I metabolites (which are not extracted by ethyl acetate) may also be present in the purified water layers (Stiborová et al. 1990a).

The highest binding of activated Sudan I was found with GMP followed by

Table 2. Binding of peroxidase-activated [^{14}C]Sudan I to nucleotides

Compound	[^{14}C]Sudan I binding to XMP (nmol.mg^{-1})	Absorbance at 480 nm
UMP	0.52 ± 0.05	0.0041 ± 0.0003
CMP	0.52 ± 0.05	0.0058 ± 0.0006
AMP	0.93 ± 0.08	0.0231 ± 0.0022
GMP	4.98 ± 0.31	0.1265 ± 0.0087

For experimental conditions see the text. The numbers in the Table represent averages and standard deviations from three parallel experiments.

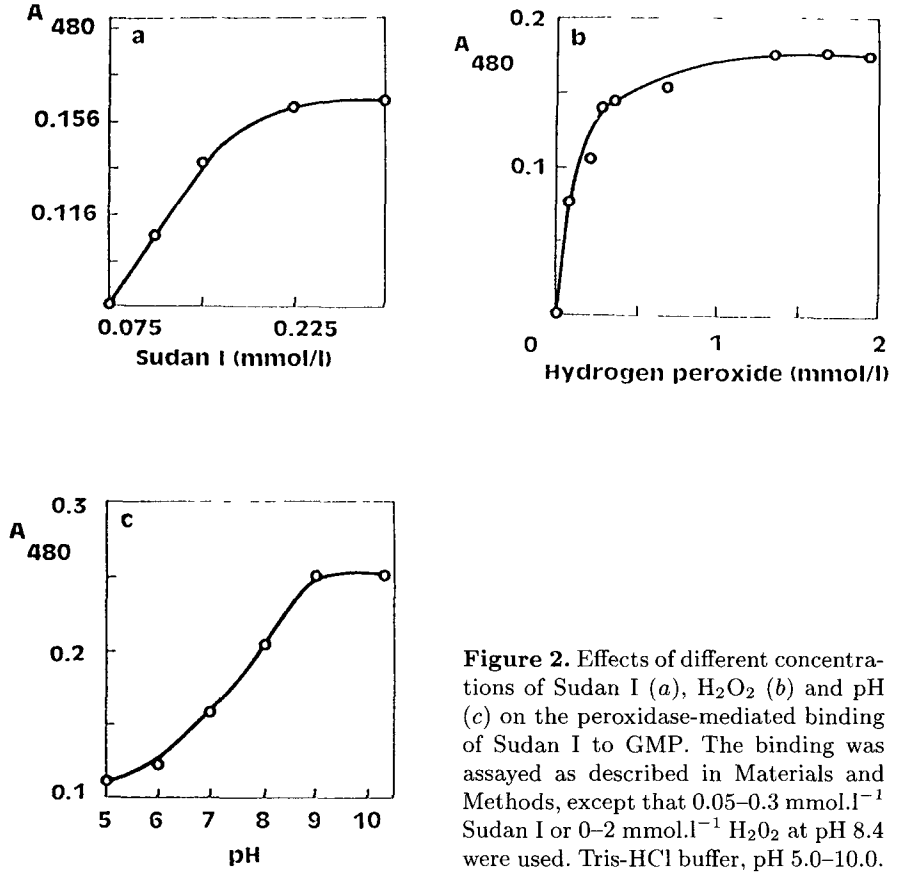

Figure 2. Effects of different concentrations of Sudan I (*a*), H_2O_2 (*b*) and pH (*c*) on the peroxidase-mediated binding of Sudan I to GMP. The binding was assayed as described in Materials and Methods, except that 0.05–0.3 mmol.l^{-1} Sudan I or 0–2 mmol.l^{-1} H_2O_2 at pH 8.4 were used. Tris-HCl buffer, pH 5.0–10.0.

AMP (Table 2). Only low amounts of Sudan I (around one tenth of the Sudan I binding to GMP) bound to UMP and CMP (Table 2). This is consistent with the binding of activated Sudan I to polyribonucleotides. As the major adduct was that with GMP (or poly G), the reaction with this nucleotide (GMP) was studied in detail.

The binding of Sudan I metabolites to GMP was dependent on the concentrations of Sudan I and H_2O_2 (Fig. 2*a* and *b*). The binding of Sudan I products to GMP showed and optimum at alkaline pH (Fig. 2*c*).

The GMP-Sudan I adduct was further isolated from the water phases of the reaction mixtures which were extracted with ethyl acetate. Excess GMP which did not react with activated Sudan I was precipitated by acetone and removed. Acetone was evaporated in a stream of nitrogen and the GMP-Sudan I adducts

present in the water phase were chromatographed on a Separon SXG C 18 (Presep) column (see Materials and Methods section) and on a thin layer of cellulose. Both chromatographic procedures are suitable for the separation of the major GMP-Sudan I adduct from residual GMP, from highly water-soluble Sudan I peroxidase mediated metabolites and from further minor GMP-Sudan I adducts

Table 3. Purification of the major GMP-Sudan I adduct by TLC on cellulose plates

TLC in *n*-butanol/ NH_4OH (14 N)/propanol/ H_2O (35 35 25 10) R_f	Products[a] obtained by incubation of [^{14}C]Sudan I, GMP and the peroxidase system	Color of compounds
0 14	2 48 ± 0 21	brown
0 23	3 81 ± 0 32	yellow-brown
0 40	5 70 ± 0 48	red
0 50	9 02 ± 0 83	orange
0 71	74 45 ± 2 91	orange (GMP-Sudan I adduct)
0 85–1 0	4 54 ± 0 43	yellow-brown (higly water-soluble products of Sudan I oxidation)

[a] ^{14}C-labeled products (adducts) were isolated and separated by TLC on cellulose as described in Materials and Methods The values shown are percentages of total radioactivity Averages and standard deviations from triplicate determinations

The major GMP-Sudan I adduct isolated by TLC on cellulose (accounting for approximately 75% of the products), R_f = 0.71 (Table 3) was characterized by UV/vis spectroscopy Similarly as poly G-Sudan I or tRNA-Sudan I adducts, the GMP-Sudan I adduct is colored and has an absorption maximum at 480 nm (Fig 3) Again, this indicates that the entire molecule of Sudan I with the azo-conjugated system binds to GMP.

GMP-Sudan I adduct is the major adduct formed in tRNA

In order to test whether the major GMP-Sudan I adduct characterized above is formed also in tRNA reacted with peroxidase-activated Sudan I, [^{14}C]Sudan I was mixed with tRNA and the peroxidase system, and purified tRNA was used for the following analysis. tRNA modified with peroxidase-activated [^{14}C]Sudan I was hydrolyzed by nuclease P1. This enzyme specifically cleaves nucleic acids to 5′-monophosphonucleosides (Fujimoto et al 1974) ^{14}C-labeled adducts obtained by

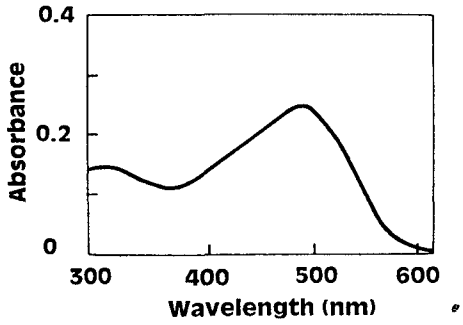

Figure 3. Absorption spectrum of GMP-Sudan I adduct (1 μg/ml) formed from GMP and Sudan I activated by the peroxidase system, and isolated as described in Materials and Methods. The spectrum was obtained in 10 mmol.l^{-1} Tris-HCl (pH 7.0) using a Perkin – Elmer Lambda 5 UV-visible spectrophotometer.

Table 4. Values of relative mobilities (Rf) of the major GMP-Sudan I adduct, and radioactivity distribution in hydrolysate of tRNA treated with [^{14}C]Sudan I and peroxidase

TLC in *n*-butanol/ NH_4OH (14 N)/propanol/ H_20 (35:35:25:10) R_f	Products[a] of hydrolysis of the [^{14}C]Sudan I-tRNA adduct	Compound
0.30	9.69 ± 0.91	unknown (colorless)
0.36	19.31 ± 1.23	unknown (colorless)
0.50	13.00 ± 1.21	unknown (orange)
0.71	51.35 ± 3.89	GMP-Sudan I (orange)
0.78	6.65 ± 0.75	unknown (orange)

[a] ^{14}C-labeled products were obtained by enzymatic hydrolysis of tRNA modified by peroxidase-activated [^{14}C]Sudan I, and separated by TLC on cellulose as described in Materials and Methods. The values shown are percentages of total radioactivity. Averages and standard deviations from triplicate determinations.

this tRNA hydrolysis were separated by TLC on cellulose. More than 50 percent of total radioactivity co-chromatographed with the major GMP-Sudan I adducts on a thin layer of cellulose with the solvent system used (R_f = 0.71) (Table 4). The UV/vis spectrum of this radioactive material obtained after elution from cellulose corresponded to that of the major GMP-Sudan I adduct (results not shown). Also, other ^{14}C radioactive zones were detected on TLC-cellulose plates (Table 4). This additional radioactive material may have represented other adducts (with guano-

sine, adenosine or other nucleosides) as suggested by the reaction of Sudan I with poly X. The results obtained strongly suggest that the major adduct derived from the reaction of activated Sudan I and GMP is also formed in tRNA.

Discussion

It has been found previously that tRNA is significantly more accessible than DNA for the binding of products of Sudan I activation by peroxidase (Stiborová et al. 1990 a,b; 1991). Moreover, a correlation was found between the modification of this nucleic acid by Sudan I (Stiborová et al. 1990 a) or other carcinogens (Fink et al. 1970; Hradec 1988, 1990; Stiborová et al. 1992) and changes in its biological activity. High levels of tRNA binding were also found with other carcinogens (i.e. 2-naphthylamine, benzidine, dimethylaminoazobenzene) (Yamazoe et al. 1985; 1988; Morton et al. 1983; Stiborová et al. 1992). Because of the high level of Sudan I binding to tRNA, this RNA could be used as a suitable model for the identification of the target nucleotides in nucleic acids for activated Sudan I.

It follows from the binding of activated Sudan I to four polyribonucleotides that poly G is the major target for reactive intermediates produced by Sudan I metabolism *in vitro*. Using the individual 5′-monophosphoribonucleosides in a detailed study, the major adduct formed from GMP and activated Sudan I *in vitro* (GMP-Sudan I adduct) was identified as the major adduct formed in the tRNA molecule. The structure of this adduct has not been identified as yet. Nevertheless, it can be postulated that this adduct is structurally different from the adduct derived from the reaction of microsomal cytochrome P-450 activated Sudan I and nucleic acids characterized previously. Recently, we have determined this adduct as being 8-(phenylazo)guanine (Stiborová et al. 1995), i.e. the adduct formed by splitting of the Sudan I molecule to benzenediazonium ion as the ultimate carcinogen. The peroxidase-mediated GMP adduct, isolated in this study, has more pronounced hydrophobic properties than the 8-(phenylazo)guanine adduct and it also differred in spectral characteristics.

Previous studies (Stiborová et al. 1990 a, b, 1991) have provided a strong evidence for the peroxidative activation of Sudan I, which leads to the formation of tRNA- and DNA- adducts, possibly involving the formation of one-electron oxidation products as the reactive intermediates. This follows from the inhibition of Sudan I-binding to nucleic acids by free radical scavengers (Stiborová and Anzenbacher 1990; Stiborová et al. 1990a,b; 1991). It could be suggested that Sudan I is primarily oxidized at the hydroxy group of its molecule to form naphthoxyradical. This suggestion is supported by the finding that a derivative of Sudan I, with the OH group acetylated (O-acetyl-Sudan I) is not oxidized by peroxidase (unpublished data). This radical or other secondary radicals which contain the whole molecule of Sudan I, can be expected to be reactive enough to attac guanosine as the nucleo-

phile in tRNA, and get bound to its molecule. Moreover, GMP-Sudan I adduct exhibited an absorption maximum at 480 nm similarly as tRNA modified by Sudan I or Sudan I alone (Stiborová et al. 1990a, 1994). This finding also strongly supports the suggestion that the entire molecule of Sudan I with its conjugated system is bound to guanosine in tRNA, being moreover responsible for the color of the modified tRNA. The identification of the adduct structure (the position of bonds in guanosine and Sudan I in the GMP-Sudan I adduct molecule) will be characterized in future. Mass and NMR spectroscopy will be used for this purpose.

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