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Urinary excretion of catechin metabolites by human subjects after red wine consumption

Jennifer L. Donovan¹*, Sidika Kasim-Karakas², J. Bruce German³ and Andrew L. Waterhouse¹

Departments of ¹Viticulture and Enology, ²Endocrinology and Metabolism and ³Food Science and Technology, University of California, Davis, California 95616, USA

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Little is known about flavonoid metabolism and excretion in man. In the present study, the urinary excretion of a major flavonoid in wine, catechin, and its metabolites, were measured after nine human subjects each consumed 120 ml red wine (RW) on one day and de-alcoholized red wine (DRW) on a separate day. Both the RW and DRW contained 120 (SEM 3) μmol catechin (35 mg). GC–MS analyses of the trimethylsilylated derivatives of catechin and 3' and 4' methylcatechin were performed before and after hydrolysis of conjugates by β -glucuronidase and sulfatase. Baseline urine samples collected prior to wine consumption contained 0.013 (SEM 0.005) μmol catechin and metabolites. During the 8 h period following consumption of RW and DRW, 6.6 (SEM 0.9) and 5.3 (SEM 0.6) μmol catechin and metabolites were excreted in 893 (SEM 94) and 740 (SEM 101) ml urine respectively. This corresponded to 3.0–10.3 % of the dose after RW and 2.1–8.2 % of the dose after DRW. The amount of catechin and metabolites excreted in urine was 20 % higher after RW compared with DRW ($P=0.06$). Catechin in all urine samples was present as metabolites and there were no differences in the proportions of individual metabolites after RW and DRW. As with other flavonoids, the fate of most ingested catechin is not yet known.

Wine: Catechin: Catechin metabolites: Urinary excretion: Man

Flavonoids are abundant components of fruits, vegetables, red wine, tea and chocolate (Kühnau, 1976; Scalbert & Williamson, 2000; Arts *et al.* 2001). They display a multitude of biological effects *in vitro* and *in vivo* after consumption of flavonoid-containing foods (Walle *et al.* 1995; Clifford *et al.* 1996; So *et al.* 1996; Teissedre *et al.* 1996; Da Silva *et al.* 1998; Nidgikar *et al.* 1998; Day *et al.* 2000; Rein *et al.* 2000a, b; Young *et al.* 2000; Schramm *et al.* 2001). Epidemiologic studies show strong associations between consumption of flavonoid-containing foods, especially wine, and reduced mortality from cardiovascular disease (St Leger *et al.* 1979; Hertog *et al.* 1993; Klatsky & Armstrong, 1993; Criqui & Ringel, 1994; Sasaki & Kesteloot, 1994; Grønbaek *et al.* 1995; Hertog *et al.* 1995; Renaud & de Lorgeril, 1992).

Little is known about flavonoid metabolism, distribution and excretion after consumption of common foods. There have been some clinical studies reporting the absorption and excretion of flavanols (Hollman *et al.* 1995, 1997; de Vries *et al.* 1998, 2001; Manach *et al.* 1998; flavones (Nielsen

et al. 1999), anthocyanins (Lapidot *et al.* 1998), flavanones (Fuhr & Kummert, 1995), flavanols (Lee *et al.* 1995; Richelle *et al.* 1999; Rein *et al.* 2000a) and isoflavonoids (Xu *et al.* 1994, 1995; Ameer *et al.* 1996) after consumption from food sources. However, the vast majority of studies were performed using gram-size doses, which gives little insight into the fate of flavonoids when consumed in smaller quantities as food constituents (Das, 1971; Das & Sothy, 1971; Gugler *et al.* 1975; Shaw & Griffiths, 1980; Shaw *et al.* 1982; Hackett *et al.* 1983; Wermeille *et al.* 1983).

The present study focused on the urinary excretion of the flavonoid catechin and its metabolites following consumption of a single serving of red wine (RW) or de-alcoholized red wine (DRW). Catechin was chosen as the flavonoid of interest because it is a major wine flavonoid and it is found in many different foods (Hollman & Arts, 2000; Arts *et al.* 2000a,b; de Pascual-Teresa *et al.* 2000). Studies have shown its potent antioxidant activity *in vitro* (Teissedre *et al.* 1996; Plumb *et al.* 1998) and its efficacy in reducing atherogenesis in an animal model (Xu *et al.* 1998).

Abbreviations: DRW, de-alcoholized red wine; 3'MC, 3'-O-methylcatechin; 4'MC, 4'-O-methylcatechin; RW, red wine containing alcohol.

* **Corresponding author:** Dr Jennifer L. Donovan, present address Laboratory of Drug Disposition and Pharmacogenetics, Department of Psychiatry, Room 246N, Medical University of South Carolina, 67 President Street, Charleston, SC 29425, USA, fax +1 843 792 6318, email donovanj@musc.edu

There are no previous reports of the urinary excretion of catechin after doses that are normally present in wine or foods. Earlier studies on the urinary excretion of catechin by human subjects used large doses of purified catechin. Das (1971) recovered 8% of a 4 g dose as catechin and its glucuronide and sulfate conjugates over 24 h, most of which were excreted during the first 6 h. Hackett *et al.* (1983) also measured the excretion of methylated catechin metabolites and reported that 48% of a 2 g dose was recovered in urine as conjugates of catechin or methylated catechin over a 24 h period.

The pharmacokinetics of catechin and its metabolites in human plasma after consumption of RW and DRW have been reported (Donovan *et al.* 1999a). In that study, catechin metabolites reached a concentration of 100 nmol/l plasma 1 h after a single serving of RW. When the results were compared with those of earlier studies that used larger doses, it was apparent that the dose influenced both the amount of catechin in plasma as well as the pattern of catechin metabolites. Few differences were observed between the amounts of metabolites in plasma after consumption of RW and DRW. Ethanol in RW, however, reduced the elimination half-life of metabolites from plasma. Thus, it is important to determine if ethanol also has an effect on urinary elimination. The present study reports the urinary excretion of catechin and its metabolites in the same human subjects over an 8 h period after RW and DRW consumption.

Methods

Chemicals and reagents

(+)-Catechin was obtained from Fluka Chemicals (Ronkonkoma, NY, USA) and (+)-taxifolin from Apin Chemicals Ltd (Abingdon, Oxford, UK). 3'-*O*-methylcatechin (3'MC) and 4'-*O*-methylcatechin (4'MC) were synthesized, purified and identified as previously described (Donovan *et al.* 1999b). The derivatizing reagent, *N,O*-bis(trimethylsilyl)-trifluoroacetamide, was purchased from Pierce (Rockford, IL, USA). β -Glucuronidase (G-0376), aryl sulfatase (S-9754) and D-saccharic acid 1-4 lactone were purchased from Sigma Chemical Co. (St Louis, MO, USA). All solvents and reagents were Fisher HPLC or Optima grade, and other reagents were either purchased from Fisher (Pittsburgh, PA, USA) or Aldrich (Milwaukee, WI, USA).

Subjects and study design

The experimental design, preparation and analysis of the RW and DRW, and the human subject selection criteria have been described previously (Donovan *et al.* 1999a; Bell *et al.* 2000). Briefly, nine volunteers (Five men, Four women, age 29 (SEM 3) years, weight 78 (SEM 4) kg consumed a flavonoid-free diet for 2 d prior to ingestion of wine. After a 14 h fast, a baseline urine sample was collected from each subject who then consumed, in random order, 120 ml either RW or DRW (100% *Cabernet Sauvignon*, 1996), providing 120 (SEM 3) μ mol of (+)-catechin (35 mg) as analysed by HPLC (Ritchey & Waterhouse, 1999). The RW contained 103 g/l ethanol and

provided 12.4 g ethanol in 120 ml. The crossover design allowed each subject to serve as his or her own control. Each subject consumed the second wine sample after resuming the flavonoid-free diet for at least 2 d. The subjects urinated as needed over the 8 h time period but were asked to empty their bladders near 8 h. The individual specimens were collected in plastic cups and the time and volume of each urine collection was recorded. A 1 ml aliquot of urine was mixed with 25 μ l phosphate-buffered ascorbic acid (0.4 mol NaH_2PO_4 /l, 200 g ascorbic acid/l, 1 g EDTA/l, pH 3.6) and was flushed with N_2 and immediately frozen at -70°C . The clinical protocol was approved by the Human Subjects Committee at the University of California at Davis.

Analysis of urine

To prevent oxidation of the analytes during sample preparation, all solvents and reagents were deoxygenated by purging with N_2 and were kept cold during extraction. The samples were thawed and 20 μ l urine were mixed with 450 μ l sodium acetate buffer (50 mmol/l, pH 5.5) and 250 μ l CaCl_2 (0.6 mol/l). Taxifolin was used as an internal standard and a final concentration of 20 μ mol/l was added to all urine samples. Samples for measurement of total catechin, total 3'MC and total 4'MC were incubated at 37°C in a shaker-water bath for 1 h in N_2 -flushed tubes containing 100 units sulfatase and 2500 units β -glucuronidase dissolved in 120 μ l water. Individual conjugate forms were determined after incubation without enzymes (free), or with β -glucuronidase (glucuronide conjugates), or with sulfatase and 0.2 mol saccharic acid 1,4 lactone/l (sulfate conjugates). Conjugates containing both the glucuronide and sulfate residues were then estimated by subtracting the free, sulfate and glucuronide forms from the total values.

After incubation, the urine was extracted with 2 ml ethyl acetate and centrifuged at 4500 g for 3 min at 4°C . The supernatant fraction was removed and the remaining fraction was extracted a second time with 1.5 ml ethyl acetate. The combined ethyl acetate extracts were passed through anhydrous sodium sulfate packed in a Pasteur pipette, dried under N_2 , redissolved in 20 μ l pyridine and derivatized with 30 μ l *N,O*-bis(trimethylsilyl)-trifluoroacetamide at 65 – 75°C for 2 h.

GC-MS analyses were performed on a Hewlett-Packard 6890 GC equipped with a 5973 quadrupole MS and a DB-23 capillary GC column (60 m \times 0.25 mm i.d., 0.25 μ m film thickness; J & W Scientific, Folsom, CA, USA). Injections of 1 μ l were made and the column temperature was programmed from 170°C (3 min hold) to 255°C at $12^\circ\text{C}/\text{min}$ and held for 12 min. The column was then cooled to 245°C at $5^\circ\text{C}/\text{min}$ and held for 6 min before the next analysis. The He carrier gas flow rate was 0.7 ml/min with an average linear velocity of 23 cm/min. Major fragmentation ions and molecular ions for catechin ($m/z = 368$ 650), 3'MC and 4'MC ($m/z = 310$ 592) and taxifolin ($m/z = 368$ 664) were monitored in selective-ion monitoring mode using a dwell time of 100 ms/channel.

For quantitation, standard curves were prepared in blank urine (i.e. urine containing undetectable levels of the analytes). Catechin, 3'MC and 4'MC were added to the blank urine at final concentrations ranging from 0.2 to

34.4 $\mu\text{mol/l}$. Taxifolin was added at a final concentration of 20 $\mu\text{mol/l}$. The urine was then incubated without addition of enzymes, extracted and analysed as described for the samples. Standard curves were linear and had average r^2 values of 0.998. The slopes of five standard curves prepared over 10 d varied $<5\%$ (CV). The limit of detection (signal: noise = 3) was 30 nmol/l for all three analytes. Reproducibility was determined by ten separate analyses of a single urine sample over a 1-month period. The method had an average CV of 11.9% for catechin, 3'MC and 4'MC.

Statistical analysis

Concentrations of catechin metabolites in urine were multiplied by the respective urinary volume to obtain the amount excreted during a given sampling time. All values are expressed as mean values with the standard errors for the nine subjects. Each subject served as his or her own control in this study and levels of catechin metabolites after RW and DRW were compared for each subject using the paired t test. To determine the changes in metabolite composition over time, the differences in the percentages of the individual conjugate forms in the first urine sample (near 1 h) were compared with the percentages in the final urine sample (near 8 h) using the paired t test.

Results

Urine volumes

Volumes of urine collected prior to RW and DRW consumption were 55 (SEM 13) and 70 (SEM 22) ml respectively. The average number of urine samples during the 8 h collection period after RW and DRW consumption was 4 (SEM 1). The total volumes of urine collected during the 8 h period after RW and DRW consumption were 893 (SEM 94) and 740 (SEM 101) ml respectively ($P=0.1$).

Total excretion of catechin

Urine samples collected before RW and DRW consumption respectively contained a total of 0.008 (SEM 0.003) and 0.020 (SEM 0.007) μmol total catechin (catechin and all measured metabolites). The cumulative excretion of total catechin in the urine of each subject over the 8 h period after RW and DRW consumption is shown in Fig. 1. The amount of total catechin in all samples increased as early as 30 min after both RW and DRW. Total catechin excreted over the 8 h period was 20% higher ($P=0.06$) after RW than after DRW. Subjects excreted a total of 6.6 (SEM 0.9) μmol after RW and 5.3 (SEM 0.6) μmol after DRW. Subjects varied in the percentage of the catechin dose excreted over the 8 h period, and values ranged from 3.0 to 10.3% after RW and from 2.1 to 8.2% after DRW. The urine samples that contained the largest quantities of total catechin were collected 2–4 h after RW and DRW consumption, containing an average of 3.7 (SEM 0.7) and 2.9 (SEM 0.7) μmol respectively.

Metabolites of catechin

The total amounts of the individual forms of catechin metabolites excreted over the 8 h period after RW and DRW consumption are shown in Table 1. The amount of free, native catechin was extremely low and always accounted for $<0.3\%$ total catechin metabolites. There were no significant differences in the percentages of the individual conjugate forms after consumption of RW and DRW.

Sulfated conjugates of catechin accounted for 40 (SEM 4)% total metabolites in urine after RW and 42 (SEM 6)% after DRW. Catechin was also excreted in a form with both glucuronide and sulfate and these accounted for 37 (SEM 4)% after RW and 35 (SEM 6)% after DRW. Only a small amount of catechin was excreted as a glucuronide conjugate.

Catechin was also methylated in the 3' position. Over the 8 h urine collection period, 19 (SEM 2) and 18 (SEM 1)% of the total metabolites were methylated in the 3' position after

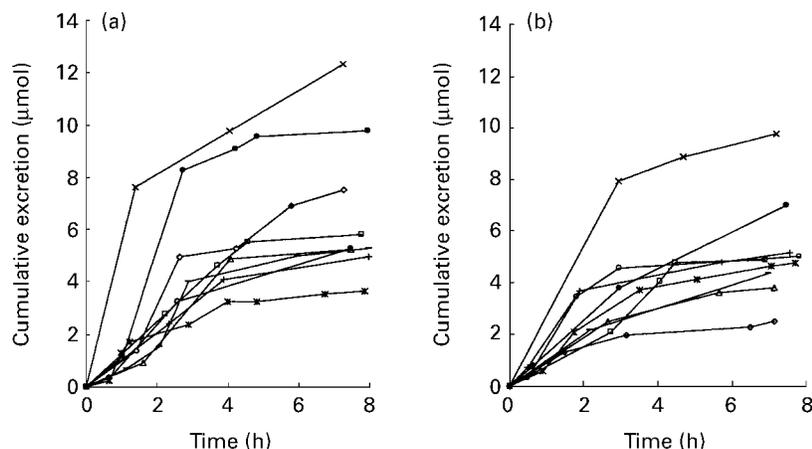


Fig. 1. Cumulative excretion of catechin and its metabolites by nine human subjects over an 8 h period following consumption of (a) 120 ml red wine (RW) or (b) 120 ml de-alcoholized red wine (DRW). For details of subjects and procedures, see p. 32. The amount of catechin and metabolites excreted in urine was 20% higher ($P=0.06$) after RW consumption than after DRW consumption.

Table 1. Conjugated forms of catechin in urine from human subjects following consumption of 120 ml red wine or de-alcoholized red wine*

(Mean values with their standard errors for nine subjects)

Treatment	Conjugate (μmol)				Conjugate (% of total)			
	RW		DRW		RW		DRW	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Metabolites†‡								
Catechin-free	0.01	0.00	0.01	0.01	0.1	0.0	0.2	0.2
Catechin-sulfate	2.86	0.65	2.38	0.55	40.2	3.5	42.1	5.5
Catechin-glucuronide	0.24	0.04	0.28	0.06	3.6	0.5	4.6	0.9
Catechin-glucuronide-sulfate	2.21	0.14	1.68	0.30	37.4	4.0	34.8	5.7
Sum of all unmethylated conjugates	5.32	0.71	4.35	0.52	81.3	1.5	81.8	1.0
3'MC-free	0.09	0.04	0.07	0.02	1.0	0.3	2.1	0.4
3'MC-sulfate	0.12	0.03	0.12	0.33	1.8	0.2	12.0	0.9
3'MC-glucuronide	0.89	0.15	0.68	0.12	13.6	1.6	12.0	1.2
3'MC-glucuronide-sulfate	0.17	0.06	0.15	0.05	2.4	0.7	3.0	1.0
Sum of all methylated conjugates	1.27	0.23	1.02	0.17	18.7	1.5	18.2	1.0
Total (all metabolites)	6.6	0.9	5.3	0.6				

RW, red wine; DRW, de-alcoholized red wine.

* For details of subjects and procedures, see p. 32.

† The total amount of catechin and metabolites excreted in urine was 20% higher after RW than after DRW ($P=0.06$).

‡ There were no significant differences in the percentages of individual conjugate forms after RW and DRW.

RW and DRW. The majority of these 3' methylated metabolites were also conjugated with glucuronide. However, smaller levels 3' methylated metabolites were also present as sulfate conjugates, glucuronide-sulfate conjugates or in unconjugated form.

Extremely small amounts of 4' methylated metabolites were also excreted in urine. An average value of 0.027 μmol were excreted after RW and an average value of 0.018 μmol were excreted after DRW over the 8 h period. These metabolites accounted for <0.5% catechin metabolites. Because the amounts of 4' methylated metabolites in urine were so low, it was not possible to determine accurately all the forms of the conjugates in all urine samples. However, in the most concentrated urine samples (obtained 2–4 h after consumption), an average of 48% catechin metabolites were present as glucuronide conjugates, 10% as sulfate conjugates and 16% as both glucuronide and sulfate conjugates.

The percentages of the conjugate forms of catechin as

well as 3'MC in the first urine samples (about 1 h) and the final urine samples (about 8 h) after RW consumption are shown in Fig. 2. The percentages of the conjugate forms in the first sample and the final sample were not significantly different.

Discussion

There is an inverse relationship between wine consumption and cardiovascular disease mortality (Renaud & de Lorgeril 1992). Ethanol in wine appears to be responsible for only a fraction of the health benefits enjoyed by wine-drinking populations (St Leger *et al.* 1979; Klatsky & Armstrong, 1993; Sasaki & Kesteloot, 1994; Gronbæk *et al.* 1995). Flavonoids in wine may provide additional protection against cardiovascular disease by acting as antioxidants (de Whalley *et al.* 1990; Fuhrman *et al.* 1995; Whitehead *et al.* 1995; Cao *et al.* 1996; Serafini *et al.* 1996, 1998; Teissedre *et al.* 1996; Hayek *et al.* 1997; Nidgikar *et al.* 1998), or

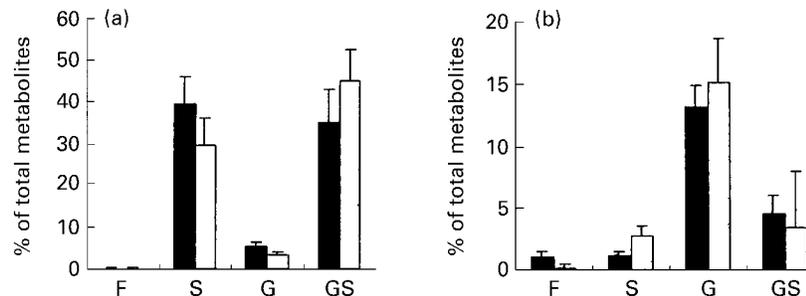


Fig. 2. Average percentages of the conjugated forms of catechin and 3'-O-methylcatechin in the first sample of urine (near 1 h) and the last sample of urine (near 8 h) following consumption of 120 ml red wine with ethanol. (a), Un-methylated forms; (b), methylated forms. Forms were free (F), sulfated (S), glucuronidated (G), or glucuronidated and sulfated (GS). ■, First urine sample; □, last urine sample. For details of subjects and procedures, see p. 32. Values are means for nine subjects with standard errors shown by vertical bars. There were no significant differences in the percentages of the conjugate forms in the early samples and the late urine samples.

reducing platelet activity and counteracting the increased activity of platelets after ethanol withdrawal (Seigneur *et al.* 1990; Demrow & Folts, 1994; Demrow *et al.* 1995; Ruf *et al.* 1995; Pace-Asciak *et al.* 1996; Renaud & Ruf, 1996; Hayek *et al.* 1997; Osman *et al.* 1998, Rein *et al.* 2000b). Flavonoids also have anti-inflammatory and vasorelaxing activities (Fitzpatrick *et al.* 1993; Schramm *et al.* 1997). Understanding the contribution of each of these mechanisms to vascular health requires comprehensive knowledge of the metabolism, distribution and excretion of flavonoids in wine. It is not known if there are additional biological effects when flavonoids are consumed in the presence of ethanol. Flavonoids and ethanol are co-ingested in wine, but similar considerations can arise from the simultaneous consumption of other sources of ethanol with foods that contain flavonoids.

We previously reported that ethanol reduced the elimination half-life of total catechin after RW consumption without affecting the pattern of catechin metabolites (Donovan *et al.* 1999a; Bell *et al.* 2000). In the present study, 20% more catechin metabolites were excreted in urine over an 8 h period after RW compared with DRW. Although the significance level was not very high ($P=0.06$), this result, coupled with our previous finding in plasma does indicate a role of ethanol in enhancing the rate of catechin elimination. The urine volume was increased by 17% after wine with ethanol suggesting that the increased elimination is due to a diuretic effect (Rozman & Klaassen, 1996). Further studies, however, are necessary to fully understand the effect of ethanol on the elimination of flavonoids as well as its mechanism of action.

Several investigators have measured the urinary excretion of other flavonoids and their conjugates after consumption of foods. In all of these studies, only a small fraction of the flavonoid dose was recovered in urine (Xu *et al.* 1994; Fuhr & Kummert, 1995; Lee *et al.* 1995; Ameer *et al.* 1996; Nielsen *et al.* 1997, 1999; de Vries *et al.* 1998, 2001; Lapidot *et al.* 1998; Watanabe *et al.* 1998; Young *et al.* 1999). Another study showed that even when absorption of flavonol glycosides is >50%, elimination in urine is <1% (Hollman *et al.* 1995).

We also found that only a small fraction of the dose of catechin is recovered in urine after RW consumption. In the present study we used a fairly short collection period, compared with other studies of flavonoid excretion. However, catechin has a much shorter half-life in plasma than other flavonoids (only 2–3 h) and most metabolites were excreted during the first 4 h (see Fig. 1). Thus, 5% of the ingested dose represents the vast majority of catechin excreted by this mechanism after wine consumption.

Differences in the proportions of specific catechin metabolites in plasma and urine indicate that certain metabolites may not be exclusively eliminated by urine. Large, extensively conjugated metabolites are more likely to be eliminated by bile (Rozman & Klaassen, 1996). Metabolites conjugated with both the glucuronide and sulfate residues made up the majority of catechin metabolites in plasma after wine consumption (Donovan *et al.* 1999a). However, these metabolites accounted for only 39% of the total catechin in urine, suggesting that biliary excretion may also be occurring. Conversely, the

metabolites that were conjugated with just sulfate were rapidly eliminated from plasma, and their increased proportion in urine indicates that these metabolites are preferentially excreted in urine.

Some catechin may also have been eliminated in forms that do not contain the intact flavonoid ring as reported in the early studies by Das (1971) and Hackett *et al.* 1983. Recent experiments also indicate that microflora present in the large intestine can metabolise catechin as well as other flavanols to smaller phenolic acids and lactones (Meselhy *et al.* 1997; Déprez *et al.* 2000; Li *et al.* 2000). These metabolites were not measured in the present study as they can originate from many phenolic components in wine. However, this may represent an important elimination mechanism that should be investigated further in the future.

The results of the present study show that after RW consumption 3–10% ingested catechin is eliminated in urine over an 8 h period. They also suggest that ethanol reduced the elimination half-life of catechin in plasma by increasing elimination in urine. Differences in the proportions of individual metabolites in plasma and urine indicate that some metabolites are not eliminated exclusively in urine. Other mechanisms of elimination, such as excretion by bile and metabolism within the large intestine must be studied further for a complete understanding of the fate of flavonoids in man.

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