Journal of Bioenergetics and Biomembranes, Vol. 37, No. 2, April 2005 (© 2005)
DOI: 10.1007/s10863-005-4132-z

In the Aging Housefly Aconitase Is the Only Citric Acid Cycle Enzyme to Decline Significantly

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Received November 15, 2004; accepted December 27, 2004

The main objective of this study was to determine if the activities of the mitochondrial citric acid cycle enzymes are altered during the normal aging process. Flight muscle mitochondria of houseflies of different ages were used as a model system because of their apparent age-related decline in bioenergetic efficiency, evident as a failure of flying ability. The maximal activities of each of the citric acid cycle enzymes were determined in preparations of mitochondria from flies of relatively young, middle, and old age. Aconitase was the only enzyme exhibiting altered activity during aging. The maximal activity of aconitase from old flies was decreased by 44% compared to that from young flies while the other citric acid cycle enzymes showed no change in activity with age. It is suggested that the selective age-related decrease in aconitase activity is likely to contribute to a decline in the efficiency of mitochondrial bioenergetics, as well as result in secondary effects associated with accumulation of citrate and redox-active iron.

KEY WORDS: Citric acid cycle; aconitase; aging.

INTRODUCTION

Aging is associated with a general decline in metabolic efficiency coinciding with deterioration in mitochondrial function (Sohal and Weindruch, 1996; Hagen *et al*., 1997). The mitochondrial citric acid cycle constitutes the energy regulating pathway of the cell where acetyl CoA is oxidized through a series of enzymatic reactions in the mitochondrial matrix (Fig. 1). The products of complete oxidation of acetyl CoA to CO_2 are the redox products, NADH and QH_2, in addition to ATP or GTP. The citric acid cycle intermediates (Fig. 1) are also the precursors for various biosynthetic pathways, such as gluconeogenesis, lipogenesis and amino acid synthesis. Therefore, the decreased efficiency of cellular metabolism characteristic of aging could result from a metabolic shift of the citric acid cycle intermediates, resulting in inefficient ATP synthesis.

Previous investigations of age-related alterations to the citric acid cycle have focused on either a single or a subset of the enzymes during the course of normal aging (Yan *et al*., 1997; Yan and Sohal, 1998; Das *et al*., 2001) or in response to exposure to exogenously-generated oxidants (Nulton-Persson and Szweda, 2001; Li and Dryhurst, 2001; Eaton and Shattock, 2002; Lee *et al*., 2003), ostensibly in the belief that enzymes exhibiting sensitivity to reactive oxygen species (ROS) would tend to be the same as those undergoing losses in activity during normal aging. Such reasoning may stem from the notion that mitochondrial deterioration during aging arises due to oxidative damage, inflicted by the ROS that were generated in mitochondria. For instance, aconitase, an iron–sulfur containing dehydratase, has been reported to be inactivated by hydrogen peroxide-induced removal of the $[4Fe–4S]^{2+}$ cluster (Nulton-Persson and Szweda, 2001), carbonylation with age (Yan *et al*., 1997; Yan and Sohal, 1998; Das *et al*., 2001), and nitrosation

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Key to abbreviations: OAA, oxaloacetate; PMS, phenylmethosulfate; DCIP, dichlorophenol-indolphenol.

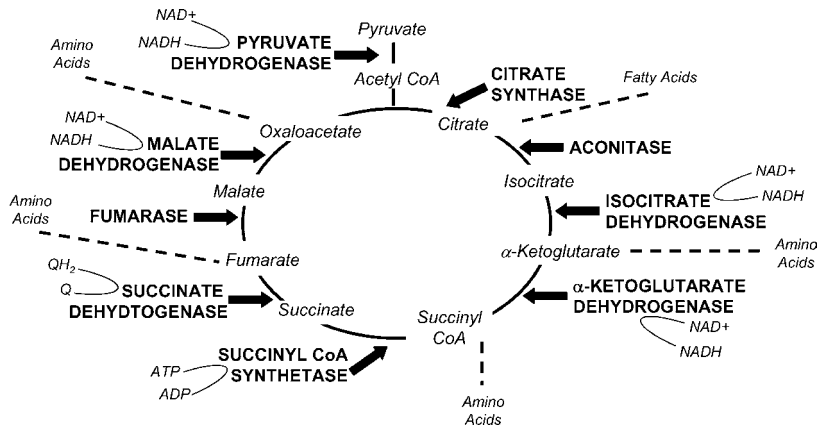

Fig. 1. A schematic drawing of the citric acid cycle enzymes, intermediates, and redox products. The citric acid cycle enzymes measured in this study are shown in capital letters. Intermediates, redox products, anaplerotic (replacing intermediates) and cataplerotic (diverting intermediates) reactions are shown in italics.

(Gardner *et al*., 1997). Pyruvate dehydrogenase and α-ketoglutarate, citric acid cycle enzymes, which directly produce NADH (Fig. 1) are inhibitable by treatment of rat brain mitochondria with DHBT-1 [7-(2-aminoethyl)-3,4-dihydro-5-hydroxy-2H-1,4-benzothiazine-3-carboxylic acid], an oxidizing agent that covalently modifies cysteine residues (Li and Dryhurst, 2001). Exposure of rat liver mitochondria with peroxynitrite, a reactive nitrogen species, results in S-nitrosylation of cysteine residues and a decrease in activity of $NADP^+$-dependent isocitrate dehydrogenase, another citric acid cycle enzyme which directly produces NADH (Lee *et al*., 2003). Malate dehydrogenase, another enzyme with NADH as the redox product, is S-glutathionylated by exposure to the photoreactive free radical-generating dye, rose Bengal, and irradiation with a white light source (Eaton and Shattock, 2002). Succinate dehydrogenase, like aconitase, is an iron–sulfur containing protein, but while aconitase is irreversibly inactivated, succinate dehydrogenase inactivation by hydrogen peroxide is reversible (Nulton-Persson and Szweda, 2001). Notwithstanding, it remains to be established whether or not such enzymes do indeed exhibit age-related losses in activity during normal aging.

Accordingly, the purpose of this study was to determine if the activities of the citric acid cycle enzymes are altered during the normal aging process. Changes in the activities of the enzymes which directly provide the reducing power, NADH and QH_2, for the electron transport complexes could directly affect energy production. In addition, an alteration of any of the citric acid cycle enzymes may alter the flux of intermediates through the cycle, resulting in inefficient energy production. The activities of each of the citric acid cycle enzymes in the flight muscle mitochondria of the housefly are reported for three different ages, corresponding to young, middle and old age.

Flight muscle mitochondria from houseflies is a convenient and reliable model system to determine the effects of aging on mitochondrial enzymes because the thoraces of houseflies are abundant in mitochondria, which also have relatively high rates of reactive oxygen species generation (Sohal and Sohal, 1991). In addition, houseflies have a relatively short life span but show a dramatic decrease in activity associated with age. Houseflies which actively fly during the first week of their life progressively show signs of being unable to lift from the bottom of their cage with time. Age-associated losses in flying ability are widely suspected to emanate from the deterioration of mitochondrial bioenergetic mechanisms, resulting in inefficient ATP synthesis (Sohal and Weindruch, 1996; Hagen *et al*., 1997). Results presented here demonstrate that the citric acid cycle is a likely contributor of the age-related deterioration of mitochondrial bioenergetics.

MATERIALS AND METHODS

Chemicals

All chemicals were purchased from Sigma (St. Louis, MO) except Triton X-100, which was obtained from Fisher. Acetyl-CoA was synthesized by dissolving the lithium salt of coenzyme A in 0.1 M Tris base to a concentration of 10 mg/mL followed by the addition of 13 μL of acetic anhydride and an overnight incubation at 4°C (Robinson *et al*., 1987).

Preparations of Mitochondria

Mitochondria from the thoraces of approximately 200 male houseflies, maintained as previously described (Bayne and Sohal, 2002), were isolated by centrifugation (Yan *et al*., 1997). The resulting mitochondrial pellet was resuspended in 30 mM potassium phosphate pH 7.0 and frozen at −80°C at a concentration of approximately 10 mg/mL. Mitochondrial pellets were diluted to 1–2 mg/mL in 30 mM potassium phosphate pH 7.0 (unless otherwise indicated) before disruption either by sonication three times for 30 s on a setting of 2 with 1 min intervals or by treatment with 0.1% Triton X-100.

Enzyme Assays

Pyruvate dehydrogenase complex was assayed by the addition of mitochondria (8.5–34 μg of protein) to the incubation mixture (50 mM Tris pH 7.8, 1 mM $MgCl_2$, 0.2 mM thiamine pyrophosphate, 2 mM sodium pyruvate, 1 mM cysteine, 0.1 mM coenzyme A, 0.5 mM NAD^+, and 0.1% Triton X-100) (Robinson *et al*., 1987). Citrate synthase activity was determined in reaction mixtures (100 mM Tris pH 8.1, 0.1 mM DTNB, 30 mM acetyl CoA, and 0.5–1.6 μg of protein) by the addition of 0.5 mM oxaloacetate (OAA) and calculated by subtracting ΔA_{412}/min before the addition of OAA from ΔA_{412}/min after the addition of OAA (Robinson *et al*., 1987). Aconitase activity was determined by using citrate as the substrate and coupling the reaction to $NADP^+$-isocitrate dehydrogenase (27 mM Tris pH 7.4, 5 mM citrate, 0.2 mM $NADP^+$, 0.6 mM $MnCl_2$, 1 unit $NADP^+$-isocitrate dehydrogenase, and 8–24 μg of protein) (Rose and O'Connell, 1967). Aconitase was also assayed using isocitrate as the substrate (90 mM Tris pH 8.0, 20 mM isocitrate, and 8–24 μg of protein) (Henson and Cleland, 1967). Mitochondrial pellets used for aconitase assays were resuspended and sonicated in 154 mM Tris pH 7.4, 5 mM citrate. For kinetic studies, the direct measurement of *cis*-aconitate formation was used by varying the concentration of isocitrate from 0.3 to 20 mM with 16 μg of protein. NAD^+-dependent isocitrate dehydrogenase activity assays (100 mM Tris-acetate pH 7.2, 1 mM $MnCl_2$, 0.5 mM ADP, 0.5 mM NAD^+, 1 mM isocitrate, and 0.1% Triton X-100) were initiated by the addition of 15–30 μg of protein (Robinson *et al*., 1987). α-Ketoglutarate dehydrogenase complex was measured by adding 13–39 μg of protein to assay mixtures (25 mM potassium phosphate pH 7.4, 0.1 mM coenzyme A, 0.2 mM thiamine pyrophosphate, 1 mM NAD^+, 0.5 mM EDTA, 5 mM $MgCl_2$, 0.1% Triton X-100, and 2.5 mM α-ketoglutarate) (Robinson *et al*., 1987). Succinyl CoA synthetase reactions (50 mM Tris pH 7.2, 110 mM sodium succinate, 10 mM $MgCl_2$, 0.1 mM ATP, 0.1 mM coenzyme A) were initiated by the addition of 15–28 μg of protein (Cha and Parks, 1964). Succinate dehydrogenase was assayed by incubating reaction mixtures (50 mM potassium phosphate pH 7.4, 20 mM sodium succinate and 15–28 μg of protein) at 30°C for 10 min, followed by the addition of 1 mM potassium cyanide, and 0.00165% dichlorophenol-indolphenol (DCIP). Reactions were initiated by adding 0.033% phenylmethosulfate (PMS) (Ackrell *et al*., 1978). Fumarase activity was determined by adding 8–24 μg of protein to mixtures (90 mM potassium phosphate and 50 mM malate) (Robinson *et al*., 1987). For kinetic studies, malate concentration was varied from 0.8 to 50 mM with 1.6 μg of protein. Malate dehydrogenase reactions (100 mM potassium phosphate pH 7.4, 0.13 mM NADH and 0.4–1.7 μg of protein) were initiated by adding 0.48 mM OAA (Robinson *et al*., 1987). For kinetic studies, OAA was varied from 7.5 to 240 μM with 0.85 μg of protein. ΔA_{340}/min before the addition of OAA was subtracted from ΔA_{340}/min after the addition of OAA.

The following extinction coefficients were used: 6.22 $mM^{-1}\,cm^{-1}$ for NADH and NADPH at 340 nm, 13.6 $\mu M^{-1}\,cm^{-1}$ for coenzyme A at 412 nm, 3.6 $mM^{-1}\,cm^{-1}$ for *cis*-aconitate at 240 nm, 4 $\mu M^{-1}\,cm^{-1}$ for succinyl CoA at 350 nm, 19.1 $mM^{-1}\,cm^{-1}$ for DCIP at 600 nm, and 4.88 $\mu M^{-1}\,cm^{-1}$ for fumarate at 250 nm.

Enzymes were assayed on two independent preparations of housefly mitochondria. Data represent averages with standard deviations of at least triplicates conducted with different enzyme concentrations. All rates were linear with respect to enzyme concentration for the amount of protein added. Kinetic data were fit with GraFit (Erithicus Software Limited). Errors in kinetic data were derived from errors of the fitting procedure provided by the GraFit software.

Mitochondrial protein concentrations were estimated by the BCA Protein Assay (Pierce, Rockford, IL) using bovine serum albumin as the standard.

RESULTS

Cross-Sectional Sampling of Flies

The average lifespan of the houseflies kept under the conditions used here varied between 16 and 20 days. Normally, the period of rapid mortality starts around 13–15 days of age. Since in an aging population, the survivors progressively represent subsets of individuals undergoing relatively slower rates of aging, cross-sectional sampling was restricted to a period prior to the onset of the dying

phase in order to obtain a representative average of the population.

Age-Related Changes in Activity of the Citric Acid Cycle Enzymes

Each of the enzymes of the citric acid cycle was assayed in mitochondria isolated from the flight muscles of the housefly at 3, 10, and 14 days of age. Aconitase was the only enzyme which showed an alteration in activity with age (Table I). Aconitase activity was measured using two different assays and was found to be decreased in old flies by approximately 44% using a direct assay, which monitors the formation of *cis*-aconitate from isocitrate (Henson and Cleland, 1967), and 30% using a coupled assay with citrate as the substrate coupled to the reaction of $NADP^+$-dependent isocitrate dehydrogenase (Rose and O'Connell, 1967). Pyruvate dehydrogenase complex, citrate synthase, NAD^+-dependent isocitrate dehydrogenase, α-ketoglutarate dehydrogenase complex, succinyl coenzyme A synthetase, succinate dehydrogenase, fumarase and malate dehydrogenase showed no age-related alterations in activity under the conditions used (Table I).

To obtain a more comprehensive pattern of age-related changes, aconitase activity was assayed at several different ages, viz., 3, 5, 7, 10, 14, and 17 days of age using the indirect or coupled assay (Rose and O'Connell, 1967) (Fig. 2). There was a relatively sharp decrease in activity between 3 and 5 days of age, followed by a more gradual decline reaching an overall level of 35% by 17 days of age. Citrate synthase activity, assayed as a control, displayed no apparent alteration in activity during the same period (Fig. 2).

Kinetics of Aconitase, Fumarase and Malate Dehydrogenase Enzyme Activity During Aging

To determine if kinetic parameters, not apparent under saturating substrate concentrations, were altered with age, activities of aconitase, fumarase and malate dehydrogenase were determined at varying substrate concentrations. Results shown in Fig. 3 indicated that, using saturating isocitrate concentration, aconitase

Table I. Activities of Citric Acid Cycles Enzymes in Mitochondria Flight Muscles of Houseflies

Enzyme	Age (days)		
	3	10	14
Pyruvate dehydrogenase complex (mmols NADH/min/mg)	0.46 ± 0.07	0.49 ± 0.08	0.45 ± 0.08
Citrate synthase (μ mols CoA/min/mg)	5.2 ± 0.2	5.0 ± 0.4	5.0 ± 0.6
Aconitase (mmols *cis*-aconitate/min/mg)	4.3 ± 0.2	2.6 ± 0.1	2.4 ± 0.1
(mmols NADPH/min/mg)	1.1 ± 0.1	0.80 ± 0.07	0.77 ± 0.06
NAD^+-Isocitrate dehydrogenase (mmols NADH/min/mg)	0.4 ± 0.2	0.4 ± 0.1	0.3 ± 0.1
α-Ketoglutarate dehydrogenase complex (mmols NADH/ min/mg)	0.69 ± 0.08	0.63 ± 0.07	0.65 ± 0.09
Succinyl CoA synthetase (μ mols succinyl CoA/min/mg)	0.9 ± 0.1	0.9 ± 0.1	0.9 ± 0.1
Succinate dehydrogenase (mmols DCIP/min/mg)	1.4 ± 0.1	1.5 ± 0.1	1.6 ± 0.1
Fumarase (μ mols fumarate/min/mg)	1.9 ± 0.2	1.8 ± 0.2	1.7 ± 0.2
Malate dehydrogenase (mmols NADH/min/mg)	26 ± 8	26 ± 5	24 ± 3

Note. Data represent averages ± standard deviations of at least triplicates conducted with different enzyme concentrations on two independent mitochondria preparations.

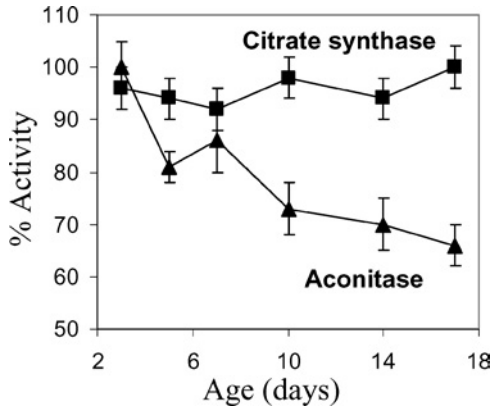

Fig. 2. Enzymatic activities of aconitase and citrate synthase in flight muscle mitochondria at different ages. Aconitase (▲) and citrate synthase (■) activities were measured at 3, 5, 7, 10, 14 and 17 days of age.

activity decreased approximately 44% between 3 ($V_{max} = 4.3 \pm 0.2$ mmols *cis*-aconitate/min/mg) and 14 days ($V_{max} = 2.4 \pm 0.1$ mmols *cis*-aconitate/min/mg). The K_m of aconitase for isocitrate did not significantly vary for the three different ages, 1.4 ± 0.3 mM at 3 days, 0.9 ± 0.2 mM at 10 days, and 0.6 ± 0.3 mM at 14 days of age (Fig. 3). The spectroscopic assay, used to determine K_m relies on measuring changes in absorbance, which are hard to differentiate at the lower substrate concentrations. As evident in Fig. 3, the V_{max} of aconitase was the kinetic feature most affected with age. The kinetics of malate dehydrogenase for oxaloacetate and of fumarase for malate were found not to be affected by aging (Fig. 3).

DISCUSSION

The results presented here demonstrate that aconitase activity decreased during aging whereas no other enzyme of the citric acid cycle showed a loss of activity. This suggests that changes in enzymatic activity during aging are not random or ubiquitous but are limited to specific enzymes. A selective age-related decrease in aconitase activity may alter the flux of the citric acid cycle intermediates, suggesting the relevance of the citric acid cycle as a contributor to the loss of efficient mitochondrial bioenergetics during the aging process.

Originally, it was widely thought that macromolecular oxidative damage during aging was ubiquitous and random due to the uncatalyzed nature of the reactions between free radicals and biological molecules (Sohal and Allen, 1990). However, it has since been shown that oxidative damage to proteins is specific rather than random (Yan *et al*., 1997; Yan and Sohal, 1998). In the present study, aconitase was found to be the only

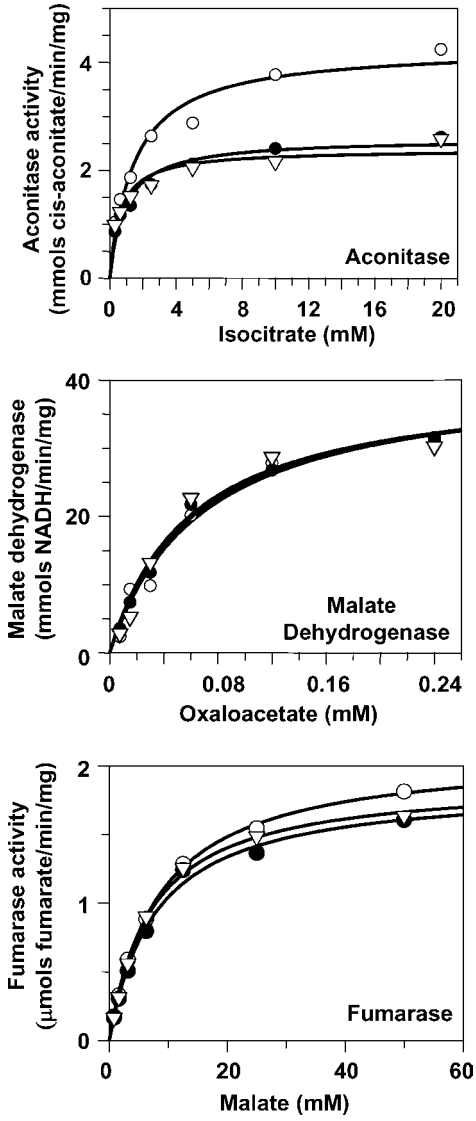

Fig. 3. Activities of aconitase, malate dehydrogenase, and fumarase under conditions of non-saturating substrate concentrations. Enzymatic activities were determined in isolated mitochondrial from 3- (○), 10- (●), and 14- (▽) day old houseflies.

citric acid cycle enzyme functionally altered during the aging process in the houseflies. This is in agreement with previous results which showed that aconitase and adenine nucleotide translocase were selectively carbonylated and functionally altered in housefly mitochondrial matrix and membrane, respectively (Yan *et al*., 1997; Yan and Sohal, 1998). An investigation of protein nitration as a consequence of biological aging has also shown selective oxidative damage in whole heart homogenate and mitochondria (Kanski *et al*., 2004). The proteins selectively targeted for nitration during the aging process

were not random, but were specifically select enzymes responsible for energy production, metabolism and maintaining the structural integrity of cells.

The age-related decrease in aconitase activity is likely to affect the flux of intermediates through the citric acid cycle, ultimately affecting the efficiency of cellular metabolism. The citric acid cycle regulates metabolic activity by not only providing the reducing equivalents, NADH and QH_2, necessary for ATP synthesis, but also the intermediates of the citric acid cycle which are used in various biosynthetic pathways, such as gluconeogenesis, lipogenesis and amino acid synthesis. Because the citric acid cycle functions in both the replacement and diversion of intermediates, an alteration of the citric acid cycle can potentially result in a variety of secondary metabolic consequences. For instance, a decrease in aconitase activity may divert the oxidation of acetyl CoA away from the subsequent production of NADH and QH_2 by the citric acid cycle towards fatty acid synthesis because a decrease in aconitase activity would lead to the accumulation of citrate, an activator of fatty acid synthesis (Fig. 1). Indeed, an age-related increase in citrate has been demonstrated in the housefly (Zahavi and Tahori, 1965) and rat (Spencer and Lowenstein, 1967). Catabolism of certain amino acids and glucose, or β-oxidation of fatty acids, which enter the citric acid cycle via acetyl CoA, may also be affected and result in less efficient energy yield. Inactivation of aconitase and the possible release of Fe^{2+} from the $[4Fe–4S]^{2+}$ cluster may lead to an accumulation of iron, resulting in increased free radical damage to macromolecules (Stadtman and Berlett, 1991). Furthermore, free iron can bind to and activate iron responsive elements in the mRNA of proteins involved in iron homeostasis and modulate their translation (Rouault, 2002).

Because cellular metabolism is composed of multiple pathways tightly coupled to the citric acid cycle, an age-related alteration of specific enzymes within multiple pathways could ultimately be responsible for the loss of locomotor ability which is widely observed in aging animals of different species. In general, the activity of the electron transport complex IV, cytochrome *c* oxidase, has been shown to be compromised with age for mammals (Kwong and Sohal, 2000; Feuers, 1998), houseflies (Sohal, 1993), and fruit flies (Morel *et al*., 1995; Schwarze *et al*., 1998). In addition, ATP levels were shown to decrease with age in fruit flies for both control and heat-stressed flies (Schwarze *et al*., 1998). However, a direct correlation was not drawn between decreased ATP levels and decreased complex IV activity because of the complexity involved with an entire organism where several pathways modulate ATP synthesis, use, and steady-state abundance (Schwarze *et al*., 1998).

In conclusion, the results of this study demonstrate that the citric acid cycle is a functional target in the aging process. Even such selective damage to the citric acid cycle can result in secondary metabolic consequences, contributing to the deterioration of mitochondrial bioenergetics.

ACKNOWLEDGMENTS

This research was supported by grant R01 AG17077 from National Institutes of Health, National Institute on Aging.

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