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Fatty acid methyl esters are detectable in the plasma and their presence correlates with liver dysfunction

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Abstract

Background: Methanol is a component of certain alcoholic beverages and is also an endogenously formed product. On this basis, we have proposed that methanol may promote synthesis of fatty acid methyl esters (FAMEs) in the same way that ethanol promotes fatty acid ethyl ester (FAEE) synthesis. We tested the hypothesis that FAMEs appear in the blood after ethanol intake.
Methods: Patient plasma samples obtained from our laboratory ($n=78$) were grouped according to blood ethanol concentrations (intoxicated, blood ethanol >800 mg/l) and non-intoxicated. These samples were further subdivided into groups based on whether the patient had normal or abnormal liver function tests (abnormal, defined as ≥ 1 abnormality of plasma alanine and aspartate aminotransferase, albumin, total bilirubin, and alkaline phosphatase). A separate set of plasma samples were also divided into normal and abnormal groups based on pancreatic function tests (amylase and lipase). There were no patients with detectable ethanol in this group. Patients with abnormalities in pancreatic function tests were included upon recognition of endogenously produced FAMEs by patients with liver function test abnormalities. FAMEs were extracted from plasma and individual species of FAMEs quantified by gas chromatography–mass spectrometry (GC/MS).
Results: Increased concentrations of FAME were found in patient samples with evidence of liver dysfunction, regardless of whether or not they were intoxicated ($n=21$, $p=0.01$). No significant differences in plasma FAME concentrations were found between patients with normal ($n=15$) versus abnormal pancreatic function tests ($n=22$, $p=0.72$).

Abbreviations: FAMEs, fatty acid methyl esters; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GC/MS, gas chromatography–mass spectrometry; amu, atomic mass unit; M14:0, methyl myristate; M16:0, methyl palmitate; M16:1, methyl palmitoleate; M17:0, methyl heptadecanoate; M18:0, methyl stearate; M18:1, methyl oleate; M18:2, methyl linoleate; M18:3, methyl linolenate; M20:4, methyl arachidonate.

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Conclusions: The presence of FAMEs in human plasma may be related to the existence of liver disease, and not to blood ethanol concentrations or pancreatic dysfunction. The metabolic pathways associated with FAME production in patients with impaired liver function remain to be identified.
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Keywords: Fatty acid methyl esters; Liver and pancreatic dysfunction; Alanine aminotransferase; Aspartate aminotransferase; Gas chromatography–mass spectrometry

1. Introduction

Methanol is a congener in nearly all-alcoholic beverages [1] and a small amount of ingested ethanol can be converted to methanol [2]. In many of our studies involving fatty acid ethyl ester (FAEE) production in various experimental models, we noted unidentified peaks close to the individual FAEE species in the gas chromatograph–mass spectrometry (GC/MS) tracings, and we speculated that these peaks could be fatty acid methyl esters (FAMEs) derived from methanol present in alcoholic beverages. Therefore, we hypothesized that an association may exist between ethanol intake and the production of FAMEs, which parallels the production of FAEEs following ethanol ingestion. The goal of this study was to determine if FAMEs are formed endogenously with ethanol intake. We tested the hypothesis using plasma samples from our clinical chemistry laboratory at the Massachusetts General Hospital (MGH) and observed that there was no relation between the presence and amount of FAMEs versus blood ethanol concentrations. Unexpectedly, however, we observed a separate association between the presence of FAMEs and abnormalities in patients' liver function tests, which did not extend to abnormalities in pancreatic function and was independent of ethanol intake. The metabolic pathway(s) by which FAMEs are generated in patients with liver dysfunction remain to be elucidated.

2. Materials and methods

2.1. Reagents and chemicals

Acetone and *n*-hexane used in this study were Optima grade (Fisher Scientific, Fair Lawn, NJ) and are suitable for use with gas chromatography–mass spectrometry (GC/MS). All fatty acid methyl ester standards including methyl heptadecanoic acid (used as an internal standard) were purchased from Nu Chek Prep (Elysian, MN).

2.2. Sample collection and extraction

This study was approved by the Institutional Review Board (IRB) of MGH. A total of 78 human plasma samples were obtained from the clinical chemistry laboratory at MGH and were separated in the following manner. Samples ($n=41$) were divided into four groups: intoxicated with abnormal liver function tests ($n=11$); non-intoxicated with abnormal liver function tests ($n=10$); intoxicated with normal liver function tests ($n=10$); and non-intoxicated with normal liver function ($n=10$). All samples were randomly collected based on blood ethanol status and whether the individual had at least one or more plasma liver or pancreatic test abnormalities. We have defined "intoxicated" as that individual whose blood ethanol concentration >800 mg/l. This matches the definition of driving under the influence (DUI) in the state of Massachusetts where this study was performed. All tracings with detectable ethanol concentrations were negative for methanol. Abnormal liver function was determined by the presence of at least one of the following plasma liver function tests outside of the reference range: alanine aminotransferase (10–40 U/l); aspartate aminotransferase (10–55 U/l); albumin (<3.0 g/dl); total bilirubin (0–1.0 mg/dl); and alkaline phosphatase (45–115 U/l).

In addition, plasma samples ($n=37$) were collected and grouped based on whether the individual had normal ($n=15$) or abnormal ($n=22$) pancreatic function tests outside of the following reference ranges as follows: amylase (3–100 U/l) and lipase (1.3–6.0 U/l).

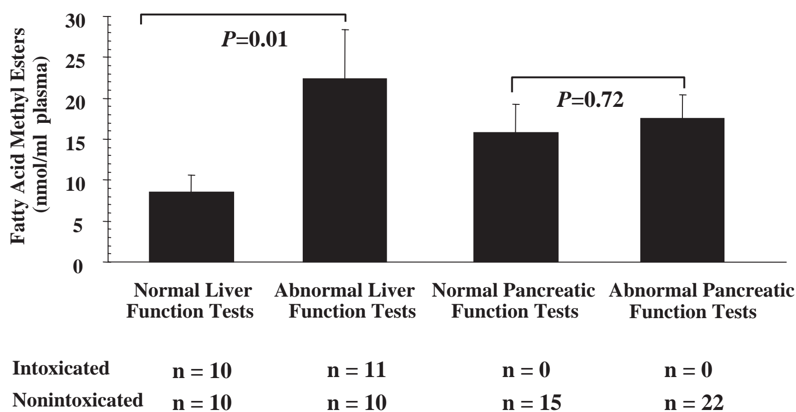

Fig. 1. Plasma FAME concentrations with or without ethanol intoxication and with normal or abnormal liver function tests. Plasma from intoxicated ($n=21$) and non-intoxicated ($n=20$) individuals with either abnormal or normal liver function tests were extracted, identified and quantified for FAME concentrations by GC/MS as described in Materials and methods. Data represent the mean ± S.E.M. of each group.

Blood ethanol and methanol concentrations for these sets of samples were all negative.

2.3. Fatty acid methyl ester extraction and quantification

Endogenous FAMEs were extracted by the addition of 4 ml of acetone to 1 ml of plasma, to which 10 nmol of heptadecanoic acid methyl ester (M17:0) was added as an internal standard. The samples were then vortex-mixed for 1 min and centrifuged at 1117 × *g* for 5 min at 4 °C in a Beckman J-B6 centrifuge. The supernatant was transferred to a separate tube. Hexane (6.0 ml) was then added to each sample. The mixture was then vortex-mixed for 1 min and centrifuged at 178 × *g* for 5 min at 4 °C. The hexane layer was transferred to a separate tube and the aqueous phase was re-extracted with an additional 2 ml of hexane. The wash was pooled with the original hexane layer and the FAMEs were concentrated by drying the sample under nitrogen vapors in preparation for injection and analysis by the GC/MS. Samples were re-suspended in 35 μl of hexane and a 1.5 μl aliquot of this was injected into a Hewlett-Packard 5890 Series II gas chromatograph equipped with a Supelcowax SP-10 capillary column (Supelco, Bellefonte, PA)

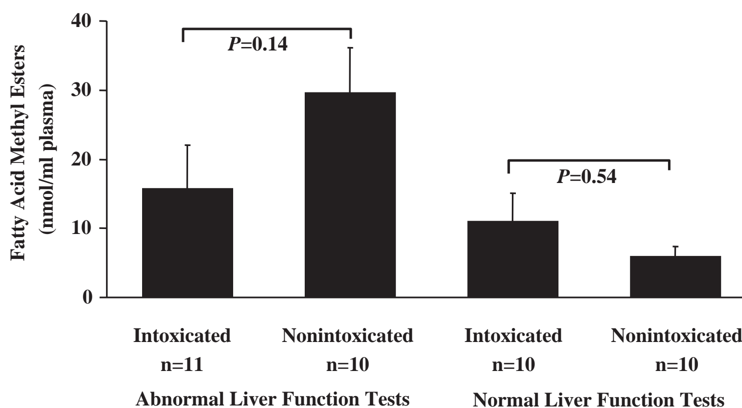

Fig. 2. Plasma FAME concentrations in patients with abnormal and normal liver and pancreatic function tests. The plasma FAMEs obtained from intoxicated and non-intoxicated patients with normal liver function tests were pooled ($n=20$). Similarly, the FAMEs obtained from intoxicated and non-intoxicated patients with abnormal liver function tests were pooled ($n=21$). Samples were extracted, identified and quantified for FAME concentrations by GC/MS as described in Materials and methods. Data represent the mean ± S.E.M. of each group.

Table 1
Blood ethanol values and liver function tests for the intoxicated, abnormal liver function test group ($n=11$)

Case #	ETOH[a] (>800 mg/l)	AST[b] (10–40 U/l)	ALT[c] (10–55 U/l)	Alk Phos[d] (45–115 U/l)	Total bilirubin (0–1 mg/dl)	Albumin (3.1–4.3 g/dl)	Relevant clinical toxicology findings
1	2628	214	129	98	1.3	3.2	ETOH abuse
2	1383	52	80	87	0.5	4.5	ETOH abuse/auto accident
3	3392	225	265	131	0.5	3.8	ETOH abuse/kidney stone/fatty liver
4	2875	52	41	77	0.2	4.1	ETOH, cocaine and benzodiazepine abuse
5	1545	36	61	97	0.5	4.8	ETOH and polysubstances abuse/auto accident
6	1637	116	103	52	0.4	4.5	ETOH abuse/auto accident
7	1424	72	31	43	0.2	3.5	ETOH abuse/auto accident
8	3173	40	83	87	0.2	4.0	ETOH, cocaine and benzodiazepine abuse
9	4968	244	ND[e]	122	0.4	3.4	ETOH abuse/hepatitis C
10	1858	173	189	112	0.2	3.8	ETOH abuse
11	5010	81	55	56	0.4	3.7	ETOH abuse/seizure

[a] ETOH, ethanol, intoxication level >800 mg/l.
[b] AST, aspartate aminotransferase.
[c] ALT, alanine aminotransferase.
[d] Alk Phos, alkaline phosphatase.
[e] ND, not done.

coupled to a HP-5971 Series 2 mass spectrometer (Agilent Technologies, Inc., Palo Alto, CA). The injector and detector temperatures were maintained at 260 °C and 280 °C, respectively. The oven program was initially maintained at 150 °C for 2 min, then ramped to 200 °C at 10°C/min and held for 4 min, ramped again at 5 °C/min to 240 °C, held for 3 min, and finally ramped to 270 °C at 10 °C/min and main-

Table 2
Blood ethanol values and liver function tests for the non-intoxicated, abnormal liver function test group ($n=10$)

Case #	ETOH[a] (>800 mg/l)	AST[b] (10–40 U/l)	ALT[c] (10–55 U/l)	Alk Phos[d] (45–115 U/l)	Total bilirubin (0–1 mg/dl)	Albumin (3.1–4.3 g/dl)	Relevant clinical toxicology findings
1	0	979	1298	98	0.8	ND[e]	CHF[f]/post liver transplantation/renal failure
2	0	536	445	87	0.4	2.6	Depression/hypothyroidism/lung disease
3	0	108	71	131	1	2.5	Thyroid cancer
4	0	549	161	77	30.8	1.0	Post liver transplantation/CAD[f]
5	0	607	574	97	6.7	2.9	Lymphoma
6	0	52	98	52	0.6	3.8	Seizure
7	0	60	38	43	0.4	4.4	Hypertension/dyspnea
8	0	23	28	87	1.4	4.4	Not known
9	0	423	355	122	2.9	3.1	Past ETOH abuse
10	0	42	101	112	3.6	2.2	Hypothyroidism/CAD[g]/rheumatoid arthritis

[a] ETOH, ethanol, intoxication level >800 mg/l.
[b] AST, aspartate aminotransferase.
[c] ALT, alanine aminotransferase.
[d] Alk Phos, alkaline phosphatase.
[e] ND, not done.
[f] CHF, congestive heart failure.
[g] CAD, coronary artery disease.

tained for 5 min. The flow rate of the helium carrier gas was maintained at a constant 0.8 ml/min throughout. Total ion monitoring was performed encompassing mass ranges from 50 to 550 amu. Peak identification was performed based upon comparison of both retention time and mass spectra of the unknown peak to that of known standards within the GC/MS database library. FAME mass was determined by comparing areas of various analyzed fatty acids to that of a fixed concentration of internal standard (methyl 17:0).

2.4. Statistical analysis

All data were expressed as the mean ± standard error of the mean (S.E.M.) for each group. All data were analyzed using analysis of variance and the unpaired Student's t-test. A $P < 0.05$ was considered statistically significant.

3. Results

In a comparison between patients with normal versus abnormal liver function tests, regardless of ethanol intoxication, there was a significant elevation in FAME concentrations when evidence of liver dysfunction was present ($p = 0.01$) (Fig. 1). Evaluation of the plasma samples of those with normal versus those with abnormal pancreatic function tests revealed no significant difference in the concentration of FAMEs between the two groups (Fig. 1). Based on the classification of intoxication alone, there was no significant difference in the plasma concentration of FAMEs between the intoxicated and non-intoxicated groups, whether or not liver function test abnormalities were present (Fig. 2). Additionally, no association was observed between FAME production and ethanol concentration measured in the patients with abnormal liver function tests (data not shown) ($r = 0.02$). FAME concentrations in the patients with normal liver function tests were also not associated with ethanol concentration (data not shown) ($r = 0.21$).

Blood ethanol concentrations (ranging from 1383 to 5010 mg/l) and liver function tests for the intoxicated individuals with abnormal liver function tests ($n = 11$) are shown in Table 1. In each case, at least one of the five liver function tests was abnormal. The most commonly abnormal tests were AST and ALT. Rele-

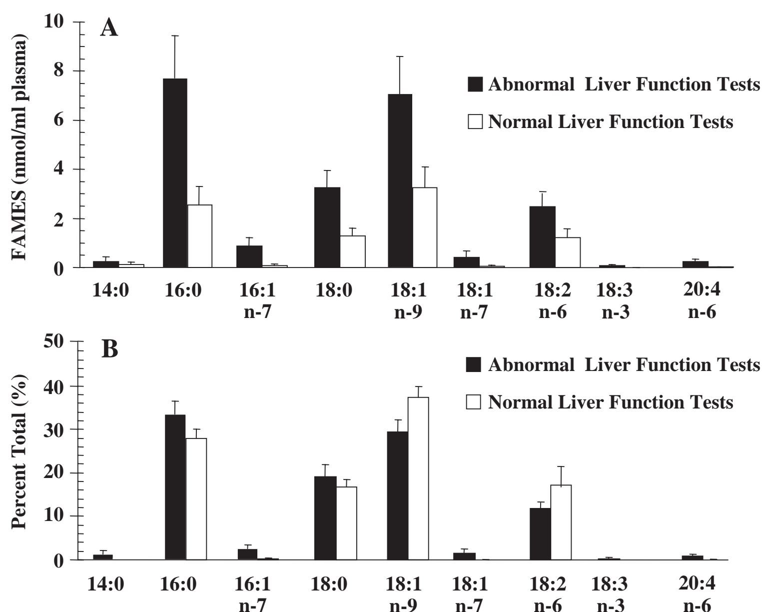

Fig. 3. The FAME compositions by mass (A) and by percentage of total of FAMEs (B) in patients with abnormal and normal liver function. Plasma from 21 intoxicated and non-intoxicated patients with abnormal liver function and plasma from 20 intoxicated and non-intoxicated patients with normal liver function were subjected to FAME isolation and identification as described in Materials and methods. The results are shown as mean ± S.E.M.

vant clinical toxicology findings showed that ethanol, cocaine and benzodiazepine were prominent drugs of abuse in these patients. Table 2 shows blood ethanol values and liver function tests for the non-intoxicated, abnormal liver function test group ($n=10$). Approximately 60% of these patients had tumors, were post liver transplantation, or had clinically severe coronary artery disease.

The distribution of FAME species (in mass, expressed as nmol FAME/ml plasma) in samples from patients with abnormal and normal liver function tests is shown in Fig. 3A. There was a significant difference in FAME masses between those with abnormal and those with normal liver function tests, especially for the saturated FAMEs, methyl 16:0 ($p=0.01$) and methyl 18:0 ($p=0.015$) and the monounsaturated FAME, methyl 18:1 n-9 ($p=0.03$). Fig. 3B shows the distribution as percentage of total FAMEs detected. These data show that the percentage differences in the distribution of FAME species are minimal. Thus, liver function test abnormalities do not result in a redistribution of FAME species, only an alteration in the amount or mass of the existing FAME species. In patients with abnormal versus normal pancreatic function tests, there were no significant differences in FAMEs observed between groups by either mass (Fig. 4A) or percentage of total FAMEs (Fig. 4B). The distribution of FAME species was similar in the liver and pancreas (Figs. 3 and 4).

4. Discussion

The presence of methanol as a marker of alcohol abuse has been suggested as a result of observations that methanol accumulates in the blood following ethanol consumption [3–7]. Blood methanol values >10 mg/l have been reported to be suggestive of alcohol abuse [8]. However, methanol is not commonly used as a marker of chronic alcoholism because its concentration in blood is much lower than ethanol during abuse. We hypothesized that an increased FAME concentration, as a result of methanol esterification to fatty acids, reflects chronic exposure to ethanol. Further, it has been shown that the liver and pancreas have the greatest FAME producing enzyme activity [1,3,9]. These findings, therefore, suggest a possible role of FAMEs in ethanol-induced organ damage since these organs are commonly affected by excess ethanol intake. However, based on the

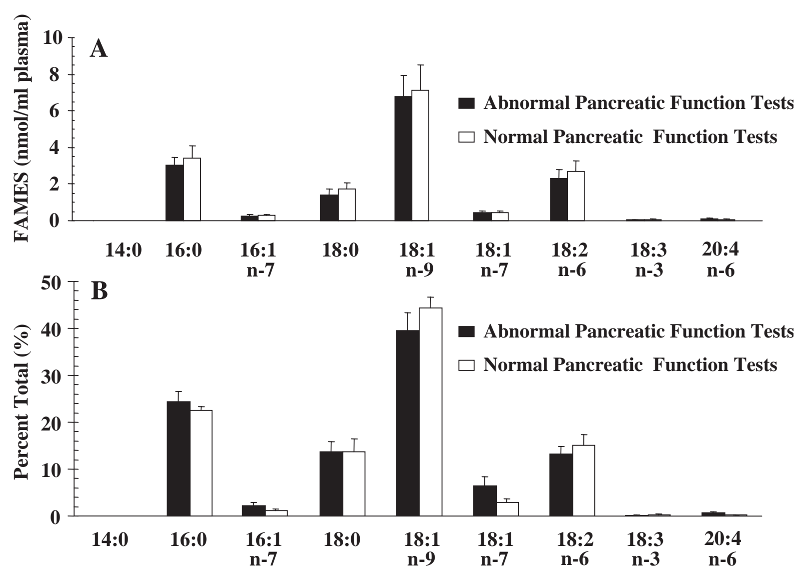

Fig. 4. The FAME compositions by mass (A) and by percentage of total FAMEs (B) in patients with abnormal and normal pancreatic function. Plasma from 22 non-intoxicated patients with abnormal pancreatic function and from 15 patients with normal pancreatic function was subjected to FAME isolation and identification as described in Materials and methods. The results are shown as mean ± S.E.M.

results of the present study, the concentration of intoxication does not appear to influence plasma concentrations of FAMEs.

Unexpectedly, however, we found that an abnormality in liver function is associated with increased concentration of FAMEs in the plasma. One of the limitations of this study is that it is impossible to know how much ethanol was ingested by the emergency department patients whose samples were analyzed, and it was impossible to know when the patients stopped drinking relative to the time of blood collection. In addition, no attempt was made to create groups of patients with specific liver or pancreatic diseases. The sample group was heterogeneous and defined by the presence of one or more abnormalities in liver or pancreatic function tests. Another limitation of this study is the possibility that the concomitant finding of FAMEs and increased ALT and AST activities does not indicate that FAME production is caused by abnormal liver function. They are associated, however, because the only consistent clinical correlation we identified for the presence of FAMEs was abnormal liver function. In addition, the elevations of ALT and AST were often not modest. The values for ALT were up to 4-fold higher (223 ± 55 U/l) than the upper limit range of normal values (10–40 U/l). Similarly, the AST values were up to 3-fold higher (210 ± 66 U/l) than the upper limit range (10–55 U/l) (Tables 1 and 2). We do not propose the use of plasma FAMEs to assess liver or pancreatic function, as the assay for FAMEs is far more complex than existing liver and pancreatic function tests.

Methanol in vivo is not derived exclusively from exogenous sources. Methanol can also be produced endogenously in the pituitary gland by the reaction between *S*-adenosylmethionine and water [10], and also as a result of the metabolism of pectin and aspartame [11–13]. This endogenous production of methanol accounts for a baseline blood concentration of 0.5–2.0 mg/l [11]. Methanol is also rapidly metabolized as blood ethanol concentrations falls to <200 mg/l due to increasing availability of alcohol dehydrogenases for methanol oxidation [6]. Approximately 96% of methanol is metabolized by alcohol dehydrogenase to formaldehyde. Formaldehyde is then rapidly converted to formic acid by formaldehyde dehydrogenase [14,15]. Detoxification of this metabolite is achieved by a tetrahydrofolate-dependent pathway, which converts formic acid to carbon dioxide and water [14].

Kaphalia et al. demonstrated increased FAME synthesis in a dose and time dependent fashion with methanol [9]. Because methanol is known to be toxic and presumably FAMEs are generated during methanol ingestion, it is reasonable to speculate that FAMEs may mediate some of the toxic effects associated with methanol intake. However, it is not known whether FAMEs possess FAEE-like toxic activity [16–19].

The biological function or toxicity and the bodily distribution of FAMEs are presently unknown. FAMEs have been found in pancreas, liver, lungs, kidney and blood [20–24]. The biological effects of FAMEs have been studied by a number of investigators. Saba et al. showed that methyl palmitate (M16:0) depressed the function of the reticuloendothelial system [25]. Methyl palmitate was also able to inactivate Kupffer cells, reducing mitogenic stimuli and inflammatory products generated by these non-parenchymal cells [26,27]. Additionally, it has been shown that inactivation of Kupffer cells by methyl palmitate prevents graft failure and improves survival rates in rats after transplantation of cold-stored livers [28]. Moreover, methyl palmitate has been found to bind to the peroxisome proliferator-activated receptor GR/NUC-1, and thereby regulate transcription [29]. In our study, the amount of methyl 16:0 in patients with abnormal liver function was significantly higher by mass than those with normal liver function ($p = 0.01$) (Fig. 3A). Several investigators have also concluded that FAMEs play a role in regulating endogenous epidermal lipids by changing the composition of fatty acids in phosphatidylethanolamine, causing epithelial hyperplasia and anticlastogenic activity, through inhibitory action of mutagens that interfere with chromosome-breaking [30–32]. Methyl linoleate (methyl 18:2 n-6) has also been found to enhance activation of the human Kv1.5 and Kv2.1 channels expressed in Chinese hamster ovary cells. Such augmentation might be physiologically related to the disease process associated with fatty acid mobilization or transportation [33].

An enzyme that promotes the synthesis of FAMEs and FAEEs has been recently purified from pancreas. It has been suggested that this purified pancreatic microsomal enzyme has broad substrate specificity towards the conjugation of endogenous long chain

fatty acids with methanol and ethanol. The purified enzyme appears to be identical to cholesterol esterase [34].

In conclusion, this study has demonstrated that the presence of FAMEs in human plasma is related to the existence of abnormal liver function and not related to the plasma ethanol concentrations.

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