Impact of polyunsaturated fatty acids on human colonic bacterial metabolism: an *in vitro* and *in vivo* study*

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Dietary polyunsaturated fatty acids (PUFA) reduce colonic proliferation and exert a mild laxative effect. We have studied the effect of the highly unsaturated eicosapentaenoic acid ethyl ester (EPA-EE) on the growth and metabolism of colonic bacteria *in vitro*, and *in vivo*. For the *in vitro* study, growth was assessed by viable counts. *Bacteroides thetaiotaomicron* was significantly inhibited in anaerobic media containing EPA-EE at concentrations > 7 g/l. *Escherichia coli* was apparently resistant even at 100 g/l. For the *in vivo* study, ten healthy volunteers ingested 18 g EPA-EE/d for 7 d. Stool frequency, 24 h stool weight and whole-gut transit time were assessed together with breath H_2 and $^{14}CO_2$ excretion following oral ingestion of 15 g lactitol labelled with 0·18 MBq [^{14}C]lactitol. The area under the breath-H_2–time curve was significantly reduced by EPA-EE, from a control value of 690·3 (SE 94·2) ppm.h to 449·5 (SE 91·7) ppm.h. Percentage dose of $^{14}CO_2$ excreted, total stool weight and whole-gut transit time were unaltered, being respectively 24 (SE 2)%, 281 (SE 66) g and 45 (SE 4) h with EPA-EE *v.* control values of 27 (SE 1)%, 300 (SE 89) g and 42 (SE 5) h. It is concluded that dietary supplementation with EPA-EE reduces breath H_2 excretion without apparently impairing overall colonic carbohydrate fermentation. The observed reduction may reflect utilization of H_2 to hydrogenate the five double bonds of EPA-EE.

Polyunsaturated fatty acids: Colonic bacteria: Eicosapentaenoic acid

Diets high in polyunsaturated fatty acids (PUFA) are being widely recommended for Western populations as part of the effort to reduce the incidence of cardiovascular disease (Wood *et al.* 1987; Oliver, 1989; Ulbricht & Southgate, 1991). The traditional Western diet, high in saturated animal fats, is also known to be associated with an increased risk of colon cancer (Nicholson *et al.* 1988). PUFA, derived mainly from plants and fish, have been shown to inhibit experimental carcinogenesis (Reddy & Sugie, 1988; Hendrickse *et al.* 1993) and colonic proliferation in patients at risk from colon cancer (Anti *et al.* 1992). Our own research (Thompson *et al.* 1990) and that of others (Czerkawski *et al.* 1966; Galbraith & Miller, 1973; Maczulak *et al.* 1981) indicates that PUFA, at concentrations as low as 10^{-5} M, exert powerful antibacterial effects in the anaerobic environments of the rumen and colon. Clinical studies currently ongoing in Nottingham as well as other institutions (Anti *et al.* 1992) indicate that diets high in PUFA can significantly reduce colonic proliferation in patients with a family history of colon cancer (Rooney, 1994). These studies also found that eight out of fifteen patients receiving eicosapentaenoic acid (EPA) developed diarrhoea (Rooney, 1994). Previous studies done in Nottingham using the highly purified ethyl ester of EPA (EPA-EE) suggested that high doses (18 g/d) of this form were also laxative (Hawthorne *et al.* 1990). One possible link between the bactericidal and laxative properties of EPA-EE is the potential reduction in colonic fermentation of unabsorbed carbohydrate.

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This would impair its degradation and subsequent absorption as short-chain fatty acids with the accompanying stimulation of Na and water reabsorption. This may increase stool weight as has been demonstrated with lactulose when its fermentation was inhibited by ampicillin treatment (Rao *et al.* 1988). Such an effect, along with the previously demonstrated stimulation of colonic motility by long-chain fatty acids (Spiller *et al.* 1986), might also contribute to the exacerbation of diarrhoea by high-fat diets in patients with small-bowel resections (Andersson *et al.* 1974), for whom colonic fermentation provides a vital enhancement of digestive capabilities.

The aim of the present study was to examine the effect of EPA-EE, the only pure form of EPA available in bulk for human use, on representative anaerobic human colonic bacteria. Having confirmed the inhibitory effects of EPA-EE *in vitro* we then set out to examine *in vivo* the effect of a high-dose of EPA-EE on the colonic salvage of ^{14}C-labelled lactitol, a nonabsorbable disaccharide (Grimble *et al.* 1988).

MATERIALS AND METHODS

In vitro *studies*

Organisms. The bacteria used in this study were *Bacteroides thetaiotaomicron* (NCTC 10582) and *Escherichia coli* (ATCC 25922). Organisms were supplied respectively by the National Collection of Type Cultures, Collindale, and the American Type Culture Collection, EuroDiagnostics, Brighton, W. Sussex. Both organisms had been stored at −70° in skimmed milk before use. During the experiments the isolates were maintained by sub-culturing every 72 h onto 70 ml/l horse-blood-agar plates. The plates were then incubated anaerobically in an atmosphere of N_2–CO_2–H_2 (80:10:10, by vol.) at 37° in a Don Whitley Mark 1 anaerobic cabinet (Don Whitley Scientific Ltd., Shipley, West Yorks). Before each experiment, absence of O_2 was confirmed by pumping gas from the cabinet through an O_2-sensitive indicator solution.

Media and reagents. Culture broth was based on the composition of ileostomy effluent (Edwards *et al.* 1986) containing (g/l): NaCl 3·51, KCl 0·75, tryptone (Oxoid, Basingstoke, Hants) 10, porcine bile extract (Sigma Chemical Co., Poole, Dorset) 5, hemin (Sigma) 0·00005, $NaH_2PO_4.2H_2O$ 1·59, Na_2HPO_4 1·39, and contained in addition 5 g/l lactitol as the carbohydrate source. Hemin (0·5 g/l solution, autoclaved at 110° for 10 min) and bile extract (50 g/l solution) were added aseptically to the rest of the medium which had been autoclaved at 121° for 20 min and then cooled. Final pH was 6·8.

Supplemented brain heart infusion (BHIS) plates were made up as follows (g/l): brain heart infusion powder (Oxoid) 37, yeast (Difco, West Molesey, Surrey) 5, hemin (Sigma) 0·005, menadione (Sigma) 0·0005, agar No. 1 (Oxoid) 10. Hemin–menadione solution (0·5 mg hemin/ml, 0·05 mg menadione/ml, autoclaved at 110° for 10 min) was added aseptically to the rest of the medium which had been autoclaved at 121° for 20 min and then cooled to 45°. The final pH was then adjusted if necessary to pH 6·8.

EPA-EE was kindly provided by Scotia Pharmaceuticals, Guildford, Surrey, with a purity of 93 %, containing in addition (mg/g); 18:4 ethyl ester 26, 20:4$n-3$ ethyl ester 10 and 20:4$n-6$ ethyl ester 26. The oil was stored in the dark at 4° to minimize autoxidation.

In vitro *study. B. thetaiotaomicron* and *E. coli* were grown under strict anaerobic conditions in batch culture using lactitol (5 g/l) as the sole C source with concentrations of EPA-EE of 0, 4, 7, 10, 40, 70 and 100 g/l in separate experiments. Pre-reduced culture medium (2·7 ml) was added in an anaerobic cabinet to pre-reduced tubes containing the EPA-EE followed by 0·3 ml of a diluted 24 h culture to give an initial viable count of 10^5 colony-forming units (cfu)/ml. Vials were sealed and crimped to exclude O_2 and removed

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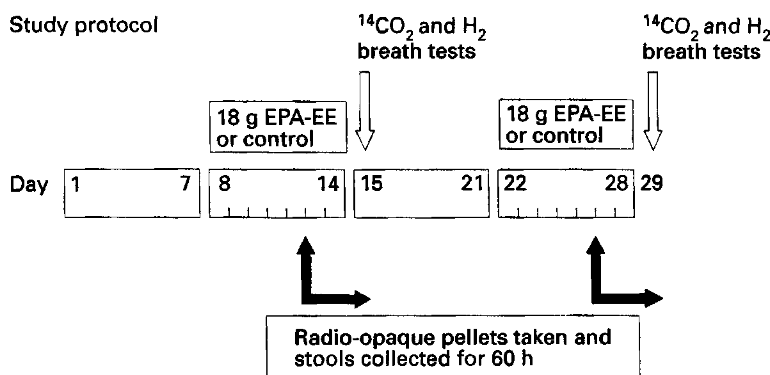

Fig. 1. The design of the present *in vivo* study. The order in which the eicosapentaenoic acid ethyl ester (EPA-EE) and control periods were undertaken was randomized, and they were separated by a 7 d washout period. Subjects kept a diary of stool movements throughout. On each study day subjects performed $^{14}CO_2$ and H_2 breath tests after first ingesting 15 g lactitol labelled with 0·18 MBq [^{14}C]lactitol.

from the cabinet. The broths were then incubated anaerobically at 37° for 16 h with shaking (150 rev./min) in a shaking incubator (New Brunswick Scientific Co. Inc., Edison, NJ, USA). Viable counts were determined at time 0 and after 16 h by dispensing 0·05 ml of a suitable culture dilution onto BHIS plates using a Spiral Systems Spiral Plater Model D (Spiral Systems, Cincinnati, OH, USA). The plates were incubated anaerobically at 37° for 24 h, in the case of *E. coli*, and 48 h for *B. thetaiotaomicron*. The resulting colonies were then counted using an AMS 40-10 image analyser (AMS, Cambridge, Cambs.).

Calculations and statistics. Growth was assessed from the $C_{16}:C_0$ ratio where C_{16} was the viable count after 16 h incubation and C_0 the viable count at time 0. In order to control for day-to-day variation in growth conditions, every experiment included six control tubes that were identical except for the omission of the substance under test. Results are reported as percentage growth relative to the mean control value giving the mean with its standard error (SEM) of six experiments. Unpaired comparisons were evalulated using the Wilcoxon–Mann–Whitney test to avoid the need to make assumptions about the normality of distribution of test results. Correlation between inhibitory effect and concentration of EPA-EE was assessed using the Spearman rank order correlation coefficient.

In vivo *study*

Study design. Ten healthy volunteers, two males and eight females, age range 21–29 years, took part in this study which was carried out over a 4-week period during which subjects recorded bowel frequency (see Fig. 1). Subjects were asked to avoid laxative foods such as curries and excessive alcohol and to take approximately 20 g fibre/d according to a diet sheet. No formal dietary surveillance was undertaken. After a 1-week run-in period, subjects either received 18 g EPA-EE at night or nothing for the study week. Then after a washout period of 1 further week subjects took the alternate treatment for a second study week, the order being randomized. No placebo was used since we were uncertain what effect other dietary oils might have. On day 5 of each study week a capsule containing twenty radio-opaque pellets was taken in conjunction with the oil and the time of ingestion recorded. All subsequent stool samples were collected in the bags provided for the following 60 h. These were sealed and labelled clearly with the volunteer's name, the date, time and number of defecation. On receipt in the laboratory samples were stored at −18° until being weighed and X-rayed. Subjects arrived fasting at 09.00 hours on day 8 (study

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day) for H_2 and [^{14}C]lactitol breath tests having taken 18 g EPA-EE last thing at night on day 7. During the control week subjects followed an identical protocol except that they took no EPA-EE. Given the small number of subjects, we were unable to stratify for age, sex and diet and we had to assume that any effect of EPA-EE would be independent of these variables.

Study day. On arrival in the Department subjects brushed their teeth thoroughly, rinsing with a chlorohexidine mouthwash. Duplicate baseline measurements for both breath tests were taken and volunteers then consumed 15 g lactitol, labelled with 0·18 MBq [^{14}C]lactitol (D-[U-^{14}C]lactitol 0·5 MBq/mmol, Amersham International, Bucks), in 150 ml water, washed down with a further 100 ml unlabelled water. After further oral hygiene, breath samples were obtained for $^{14}CO_2$ and H_2 assay every 30 min for the first 2 h and then hourly for the following 5 h, by which time breath H_2 was approaching baseline values. During the study day the subject's activities were restricted to either sitting in a chair or reclining on a bed. They were given a standard 2510 kJ sandwich lunch and allowed unrestricted fluid intake. Smoking on the study day was forbidden and subjects were requested not to indulge in excessive amounts of beer or hot spicy foods, e.g. curries, for the entire study. They were also asked to avoid lentils and beans the night before each study day in order to obtain a low baseline breath H_2. Antibiotic consumption in the 4 weeks before and during the study was also an exclusion.

Breath analysis: hydrogen. Samples (20 ml) of end-expiratory air were collected by standard techniques (Metz *et al.* 1976) and analysed using an exhaled H_2 monitor (GMI Ltd., Renfrew, Scotland). H_2 production was assessed from the area under the concentration–time curve for the 7 h of study. Mouth to caecum transit was assessed from the time taken for breath H_2 to rise to 50% of maximum rise (T_{50}) and from the time to peak (T_{peak}).

[^{14}C]lactitol breath test. CO_2 (1 mol) was trapped in hyamine hydroxide using standard techniques (Kaihara & Wagner, 1968) and the amount of $^{14}CO_2$ assessed by scintillation counting using a LKB Wallac 1215 Rackbeta 11 liquid scintillation counter (Wallac Oy, Turku, Finland). $^{14}CO_2$ excretion was expressed as a percentage of the administered dose excreted per h. A graph of percentage dose excreted *v.* time was plotted and the area under the curve (AUC) calculated.

Stool weight, frequency and mouth to anus transit. The frozen stool samples were weighed and then X-rayed to allow the number of plastic pellets to be counted. The cumulative excretion of pellets was plotted against time. The measure of transit time used was the time taken for 25% of the ingested pellets to be excreted (not all subjects excreted 50% of pellets within 60 h).

Statistical analysis. Paired comparisons were made using the non-parametric Wilcoxon signed rank test except for analysing whole-gut transit when the rank sign test (Siegel & Castellan, 1988) was used because in five out of eighteen cases transit time was $>$ 60 h and the two observations on each subject were therefore ordinal only.

RESULTS

In vitro *study*

B. thetaiotaomicron grew well under control conditions, counts rising from initial values of 1×10^5 to 1×10^7 within 16 h. Growth was however markedly inhibited by EPA-EE, an effect which was strongly correlated with the concentration of EPA-EE (Fig. 2). Pure non-esterified EPA was significantly more potent at a concentration of 10 g/l, reducing growth to 27 (SE 5)% of control compared with the 48 (SE 5)% observed with 10 g EPA-EE/l (Fig. 2, $P < 0.01$, *n* 6). In keeping with previous experiments, the growth of *E. coli* was not

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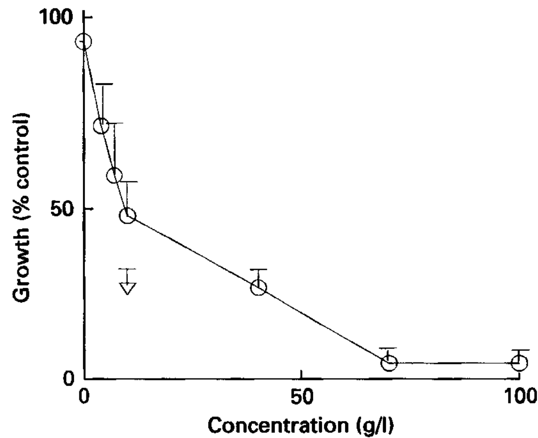

Fig. 2. The effect of eicosapentaenoic acid (EPA; ▽) and EPA ethyl ester (EPA-EE; ○) on the growth of *B. thetaiotaomicron in vitro*. The figure shows an inverse log-linear relationship between concentration of EPA-EE and growth, expressed as a percentage of the control. The Spearman rank order correlation coefficient was highly significant at 0·985 ($P < 0{\cdot}001$, *n* 6). The single data point with EPA showed significantly greater inhibition at 10 g/l compared with EPA-EE ($P < 0{\cdot}01$, *n* 6), suggesting that the free acid was the active form. Values are means with their standard errors represented by vertical bars.

Table 1. *Effect of eicosapentaenoic acid ethyl ester (EPA-EE; 18 g/d) on the colonic fermentation of 15 g [^{14}C]lactitol in nine healthy volunteers**

(Mean values with their standard errors)

	Control		EPA-EE		Difference:
	Mean	SE	Mean	SE	EPA-EE *v.* control
Breath H_2					
Area under curve (ppm.h)	690	94	450	92	$P < 0{\cdot}02$
Time to peak (h)	3·1	0·4	3·2	0·3	NS
$^{14}CO_2$					
% excreted at 7 h	27	1	24	2	NS
Peak excretion (% dose/h)	5·5	0·3	6·3	0·3	NS
Stool					
Weight (g/3 d)	300	89	281	66	NS
Frequency (stools/7 d)	8·2	2·2	8·1	2·2	NS
Time to excrete 25% markers (h)	42	5	45	4	NS

* For details of subjects and procedures, see pp. 735–736.

significantly affected by the addition of EPA-EE, even at a concentration of 100 g/l, growth being 96 (SE 4)% of control. Pure EPA at 10 g/l was likewise without effect, growth reaching 97 (SE 6)%.

In vivo *study*

Nine volunteers complied fully with the study, one subject being excluded for failing to take the oil. All were asked about diarrhoea but surprisingly only one reported loosening of stool while taking EPA-EE.

Breath hydrogen test. All subjects showed a marked rise in breath H_2 after 10 g lactitol, peaking at 2–5 h and declining thereafter. Eight out of nine subjects demonstrated a decrease in H_2 excretion when taking EPA-EE (Table 1), with AUC decreasing by

240 (SE 94) ppm.h ($P < 0.02$). No obvious change was observed in either the time of arrival of the head of the lactulose bolus as assessed by T_{50} (0·9 (SE 0·3) control *v*. 1·1 (SE 0·2) h with EPA-EE), or the time for the bulk of the lactitol to reach the caecum as assessed by T_{peak} (Table 1).

[^{14}C]lactitol breath test. All subjects showed a significant rise in $^{14}CO_2$ excretion which EPA-EE did not appear to alter. Excretion of $^{14}CO_2$ over the 7 h experiment was not significantly different from the control value, nor was there any significant change in the time to reach peak excretion or the mean excretion assessed as percentage dose/h. Although both curves were declining, breath $^{14}CO_2$ was still well above baseline at 7 h, when subjects were allowed home.

Stool weight, frequency and mouth to anus transit. Stool weight showed marked interindividual variation with the two vegetarians having much heavier stools. These differences were consistent and treatment with EPA-EE had no statistically significant effect on total stool weight or cumulative number of defecations over 7 d. The mean mouth to anus transit time was likewise unaltered (Table 1).

DISCUSSION

Our *in vitro* studies confirmed that EPA-EE significantly inhibits the growth of the obligate anaerobe *B. thetaiotaomicron* at concentrations as low as 10 g/l (30 mmol/l). The growth of the facultative anaerobe *E. coli*, however, was unaffected at concentrations of 100 g/l. These findings are in accordance with our previous findings that PUFA exert significant inhibitory effects on anaerobic organisms, whilst the growth of *E. coli* is unaffected (Thompson *et al.* 1990). Our finding that the free EPA showed an even more pronounced inhibitory effect compared with EPA-EE suggests that the ester must be hydrolysed before it exerts its full effect. Similarly, when the bactericidal properties of free linoleic acid on the growth of *Helicobacter pylori* were compared with the neutral triacylglycerol trilinolenin it was apparent that it was the free fatty acid which was the active moiety (Thompson *et al.* 1992).

The mechanism of inhibition of these anaerobic bacteria by PUFA is at present unknown, however electron-microscopic studies of other Gram-negative organisms have shown that the outer membrane is disrupted (Thompson *et al.* 1992) and other authors have also suggested that the outer membrane is the site of action of PUFA (Galbraith & Miller, 1973).

Although PUFA appear to be absorbed well, with an efficiency similar to (Chernenko *et al.* 1989) or slightly less than oleic acid (Chen *et al.* 1987), there is some evidence that EPA-EE may be somewhat resistant to pancreatic lipase (*EC* 3.1.1.3) (for review see Nelson & Ackman, 1988). This is supported by the slower rate of appearance in plasma lipids of EPA given as the ethyl ester compared with both the triacylglycerol, dioctanoyl-2-eicosa-pentaenoyl glycerol, and the free EPA (El Boustani *et al.* 1987). Assuming a poorer absorption than saturated fats, 5–10% EPA-EE would be predicted to escape absorption in the small intestine. An intake of 18 g would therefore give > 1.8 g to be diluted in 100–200 g stool/24 h, providing a concentration of about 10 g/l which we have shown significantly inhibits growth of colonic anaerobes. Supporting this concept, many other dietary studies have shown appreciable (1–5 mmol/l) (Govers & van der Meer, 1992; Lapre *et al.* 1993) concentrations of dietary fatty acids in stool water.

Our experiments used a pure culture of a single bacterial type whereas the colon contains more than thirty-seven different bacterial species, each one of which is capable of adapting its metabolism to new conditions. It follows therefore that any conclusions derived from a

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simple pure culture model require testing and subsequent validation *in vivo* where inhibiting one species may simply cause an increase in another less susceptible species.

Our *in vivo* study was an attempt to determine whether conclusions derived *in vitro* could be used to predict the effect of dosing with EPA-EE on colonic salvage of lactitol. EPA-EE was generally well tolerated, with only one subject complaining of looseness of stool. We found that 18 g EPA-EE for 7 d reduced total breath H_2 excretion without significantly altering frequency of defecation, colonic transit time or stool weight. Furthermore, the excretion of $^{14}CO_2$ derived from ^{14}C-labelled lactitol remained unchanged, implying that the processes underlying 'colonic salvage' (Bond *et al.* 1980), that is fermentation, absorption of short-chain fatty acids and their subsequent metabolism, were also unaltered. This accords with the lack of change in stool weight. Our method for assessing whole-gut transit time was a crude, single-point estimate which is unreliable compared with that obtained using multiple markers over longer study periods. However, this was not the main focus of the present study and the method was designed simply to rule out any substantial acceleration in transit. Minor changes in transit would not account for the reduction in breath H_2 production observed since the lactitol fermentation in both cases appeared to be substantially completed within 7 h, when almost none of the plastic markers had been excreted. We chose not to use a placebo control as we were uncertain whether other oils might have similar effects. However, the observed differences were substantial and it is hard to see how knowledge of whether subjects were taking an oil or not could have produced such changes, since the breath H_2 readings were highly reproducible.

Breath H_2 derives from the anaerobic fermentation of carbohydrate which produces a surplus of reducing equivalents from the oxidation of pyruvate (Miller & Wolin, 1979). These can be used in a number of ways including the reduction of CO_2 to produce CH_4 (Strocchi *et al.* 1991), and SO_4 to produce H_2S (Christl *et al.* 1992*a*). Changes in the colonic flora, such as after broad spectrum antibiotics, can alter H_2 production (Rao *et al.* 1988) while providing competing substrates such as sulphate (Christl *et al.* 1992*a*) can significantly alter this process by competing for H_2 and thus lowering CH_4 production. EPA-EE could, in the study presented, act in both ways. As we have shown, it does have selective antibacterial properties so it may well alter colonic flora, for example by favouring facultative aerobes like *E. coli*, which tend to ferment carbohydrates such as mannitol without H_2 production. Treon *et al.* (1989) have shown that diets high in marine oils lead to a reduced anaerobe:aerobe ratio in the colon which might well alter carbohydrate fermentation. EPA-EE also has five double bonds which are highly likely to compete for H_2 as they become saturated during bacterial metabolism. The anaerobic bacteria of the sheep rumen rapidly saturate PUFA (Noble *et al.* 1974) thereby markedly reducing the production of CH_4 which is the alternative pathway for excretion of excess reducing equivalents (Czerkawski *et al.* 1966). If 1·8 g EPA-EE were fully saturated this would consume 612 ml H_2/d. This represents a substantial proportion of most individuals' H_2 excretion and would be predicted to reduce substantially the response to lactitol as we found (Christl *et al.* 1992*b*).

We conclude that EPA significantly alters colonic bacterial fermentation, which is a major determinant of the composition of colonic contents. This needs to be considered when interpreting some of the observed effects of PUFA on colonic proliferation and bowel habit (Anti *et al.* 1992; Rooney, 1994). Plainly, further research into these colonic effects is warranted before we recommend far-reaching changes in the population's diet which may have important, but unknown, effects on colonic metabolism.

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