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MONOGLYCERIDE TRANSACYLASE OF RAT-INTESTINAL MUCOSA

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SUMMARY

1. Monoglyceride transacylase occurs in the small intestinal mucosa of the rat in two forms; one is a soluble protein in the cell sap, while the other is tightly associated with subcellular structures such as mitochondria and microsomes.

2. A partially purified particulate monoglyceride transacylase of rat small-intestinal mitochondria is studied with respect to cofactor requirement, pH optimum and substrate specificity using various monoglycerides. Palmitic acid, ATP and CoA can be replaced by palmityl CoA.

3. The reaction product is identified as diglyceride.

INTRODUCTION

It is well established that relatively large amounts of monoglyceride arise as products of triglyceride digestion in the lumen of the small intestine[1-8]. After absorption and passage through the mucosal cells, the fats containing long-chain fatty acids are transported via the chyle[9-11]. Triglycerides may make up as much as 90% of the total chyle lipids[12-14], and thus monoglycerides are either hydrolysed after absorption or utilised as a unit, for the synthesis of triglycerides.

Feeding experiments with glycerides containing α-dimethyl derivatives of long-chain fatty acids[15] indicated that the intraluminar digestion of triglycerides does not go to completion and that the monoglycerides formed were absorbed and used as a unit for the resynthesis of chyle triglycerides. The possibility of a direct esterification of monoglycerides has been pointed out by FRAZER[16] and later by KENNEDY[17].

Experimental evidence for the occurrence of a monoglyceride transacylase was first obtained with subcellular preparations of the small intestinal mucosa of the rat and rabbit[18-20]. Subsequently soluble and particulate preparations of other mammalian organs were also shown to catalyse this reaction[21]. The direct esterification of monoglycerides catalysed by particulate preparations of rat small-intestinal mucosa was recently confirmed by using glycerol-labelled monopalmitin and palmityl CoA[22].

In this communication, some further properties of the particulate monoglyceride transacylase from the mucosa of the small intestine of the rat are described. A preliminary report of this work has been published[23].

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MATERIALS AND METHODS

Methods and materials not described below have been published[19].

Pancreatic lipase, bile salts, α-glycerophosphate, monocaprin, monocaprylin, monoundecalin, monoheptylin, monolinolein, and monoricinolein were commercial preparations.

Preparations and purification of monoglycerides

α-Monostearin was synthesised by the method of BAER AND FISCHER[24]. The glycerol content of the preparation was 25.2% by weight (theory 25.6%).

The commercial monoglyceride preparations were separated from free fatty acids by chromatography on alumina as follows: Petroleum ether (40–60°) solutions were applied to alumina columns made up in the same solvent. After washing the columns with 10 column volumes of carbontetrachloride, the monoglycerides were eluted with 10 column volumes of chloroform. With some of the monoglycerides it was necessary to carry out a further purification by silicic acid chromatography according to BARRON AND HANAHAN[25]. Each of the purified monoglycerides was finally crystallised from *n*-hexane at −20°.

Preparation of monoolein and α,β-diolein

These were prepared by enzymic hydrolysis of olive oil by pancreatic lipase. The incubation mixture consisted of 25 ml of olive oil, 65 ml of 0.066 M phosphate buffer (pH 7.4), 780 mg of pancreatic lipase in 13 ml of water, 0.5 ml of a 10% solution of bile salts and 20 ml of 1 M $CaCl_2$. The reaction mixture was shaken for 5 h at 37°. At the end of the incubation period 60 ml of petroleum ether (40–60°) were added and the lipids extracted. The extraction was repeated twice with the same amounts of petroleum ether. The combined extracts were washed with water, filtered and taken to dryness under reduced pressure at 35–40°.

The glycerides and free fatty acids were then taken up in chloroform, applied to an alumina column made up in chloroform, and elution was carried out with 5 column volumes of chloroform. The glyceride-containing eluate was freed from chloroform by distillation at reduced pressure. A separation of the lipid into tri-, di-, and monoglycerides was achieved by chromatography on silicic acid[25].

Analysis of the glycerides

Ester determination was carried out according to STERN AND SHAPIRO[26]. The glycerol content was estimated using the method of BURTON[27] after hydrolysis of the glycerides in 0.5 N KOH for 4 h at 37°. The α-isomer of the monoglycerides was determined using a modification[28] of the method of POHLE AND MEHLENBACHER[29]. The results of the analyses are given in Table I.

Preparation of [^{14}C]palmityl CoA.

The thioester was prepared enzymically using a partially purified thiokinase from rat liver.

The thiokinase was prepared as follows: Six rat livers were homogenised in 200 ml of 0.066 M phosphate buffer (pH 7.4). The homogenate was centrifuged at 105000 × *g* for 60 min and the supernatant fluid was collected. Solid ammonium

sulphate was added to give a final concentration of 45% and the precipitate collected by centrifugation at 10000 × *g* for 10 min and dissolved in 0.066 M phosphate buffer (pH 7.4) (about 30 mg of protein/ml). Enough ammonium sulphate was added to give a final concentration of 25%, the precipitate was collected as above and dissolved in 0.066 M phosphate buffer (pH 7.4) (about 20 mg of protein/ml). This was stored

TABLE I

ANALYTICAL DATA OF THE MONOGLYCERIDE PREPARATIONS

For details of analytical methods see text.

Monoglyceride	*Molar ratio of glycerol:ester*	*Percentage of α-monoglyceride*
Monoacetin	1:0.81	—
Monocaprin	1:1.16	33.6
Monocaprylin	1:0.98	63.0
Monoundecylin	1:0.93	75.0
Monoheptylin	1:1.03	70.0
Monoricinolein	1:1.02	27.4
Monolinolein	1:0.94	74.0
α-Monoolein	1:0.96	94.0
Monoolein	1:1.01	60.0
α-Monopalmitin	1:1.06	100
α-Monostearin	1:1.10	100

overnight at −8°, thawed, centrifuged for 60 min at 105000 × *g*, and the sediment suspended in phosphate buffer using a glass homogeniser. This final preparation (about 20 mg protein/ml) represented a 7–10-fold purification over the first extract and catalysed the formation of 1.8–2.4 μmoles of thioester/h/mg protein using palmitate as substrate. It had no lipase activity and contained no fatty acid esters[26]. Enzymic activity decreased markedly upon storage.

For the formation of palmityl CoA, 200 μmoles of [^{14}C]palmitate (containing 50 μC) were added to 200 μmoles of ATP, 20 mg of CoA (70–75% pure), 320 μmoles of $MgCl_2$, 200 μmoles of glutathione, 1.6 mmoles of KCl, and purified thiokinase in a total volume of 30 ml. The pH was adjusted to 7.4 with a few drops of 2 N KOH and incubation was carried out at 37° for 1 h. The reaction was stopped by the addition of 3 ml of 50% trichloroacetic acid. The mixture was quickly cooled to 0° and then centrifuged. The sediment was washed with 40 ml of the following: ice-cold 5% trichloroacetic acid (twice), distilled water (twice), ice-cold acetone (once), and ethyl ether at room temperature (five times). Palmityl CoA was extracted from the residue four times with 10-ml portions of isopropanol – pyridine – water (1:1:1, v/v) in a water bath at 40°. The combined extracts were taken to a smaller volume under reduced pressure in a water bath at about 40° to remove most of the alcohol and the remainder was freeze-dried. The white solid was dissolved in 4 ml of water, giving a turbid solution.

The solution contained 61.5 μmoles of organic phosphate and 19.7 μmoles of ester as determined by the hydroxamic acid method[30], thus giving an almost theoretical yield when related to the CoA added. In a control experiment, it was shown that in the absence of CoA, the freeze-dried isopropanol–pyridine–water extract contained less than 0.5 μmole of organic phosphate and no carboxylic acid esters.

The ratio of the absorbancies of a solution of the palmityl CoA at 258 and 230 mμ was 1.72. This agrees with a value of 1.80 calculated from the results of SRERE, SEUBERT AND LYNEN[31] for their preparation of palmityl CoA and is very different from the value of 3.38 given by free CoA. The preparation contained no free sulphhydryl groups as determined by the dye reduction method of BASFORD AND HUENNEKENS[32]. In the presence of crude tissue extracts which are known to contain an acyl CoA deacylase[31] a linear increase in the amount of free sulphhydryl groups with time was obtained.

Adult albino rats of either sex were used throughout this work. They were not starved before death.

EXPERIMENTS AND RESULTS

Intracellular distribution

The fractionation of the homogenate from rat small-intestinal mucosa was carried out in 0.25 M sucrose[33]. Most of the mucus was removed by filtering the homogenate through lint. The mitochondrial preparation was further fractionated into a soluble extract and a sediment by freezing and thawing it twice in 0.066 M phosphate buffer (pH 7.4) followed by centrifugation at 17000 × *g* for 15 min, since preliminary experiments had shown that the sediment retained all the esterifying activity of the original mitochondrial fraction. Each subcellular fraction was tested for its ability to esterify monoolein with [^{14}C]palmitate (see Table II).

TABLE II

MONOGLYCERIDE TRANSACYLASE IN SUBCELLULAR FRACTIONS OF RAT-INTESTINAL MUCOSA

The assay system consisted of 1.0 ml of subcellular preparation (5 mg protein in 0.066 M phosphate buffer, pH 7.4, except the supernatant fraction which was in 0.25 M sucrose), 20 μmoles of ATP, 0.25 μmoles of CoA, 10 μmoles of glutathione, 20 μmoles of KF, 1.0 μmole of [^{14}C]palmitate and 10 μmoles of monoolein. The final volume was made up to 3.0 ml with deionised water, and the tubes were incubated for 60 min at 37°. Specific activity refers to mμmoles of [^{14}C]palmitate incorporated/h/mg of protein. In control experiments, ATP and CoA were omitted and only the energy-dependent incorporation of palmitate was taken into consideration for the above quoted values.

Cell fraction	*Total protein (mg)*	*Specific activity*	*Per cent of total activity*
Mitochondrial sediment	450	0.60	21
Mitochondrial extract	553	Nil	Nil
Microsomal fraction	719	0.25	13
Supernatant	1515	0.57	66

There was a wide distribution of activity throughout the cell, the largest percentage of the total activity being in the cell sap. The specific activity of this fraction was about the same as that for the mitochondrial sediment. Although it contained half of the original mitochondrial protein, the mitochondrial extract had little or no activity. A recombination of the mitochondrial extract with the sediment did not bring about an increase in the amount of palmitate incorporated by the sediment alone. The mitochondrial sediment was used for further studies on the monoglyceride transacylase.

Requirement for monoolein

Raising the concentration of monoolein in the presence of ATP, CoA, and GSH resulted in an increased incorporation of palmitate (see Fig. 1). The optimal concentration of monoolein was 3.3 mM. Monoolein could not be replaced by free glycerol and in most experiments α-glycerophosphate or α,β-diolein were less active than the monoglyceride (see Table III, Expt. 3).

Requirement for cofactors

The cofactor requirement was investigated by using either pure α-monoolein or a monoolein containing 60% of the α- and 40% of the β-isomer (see Table III).

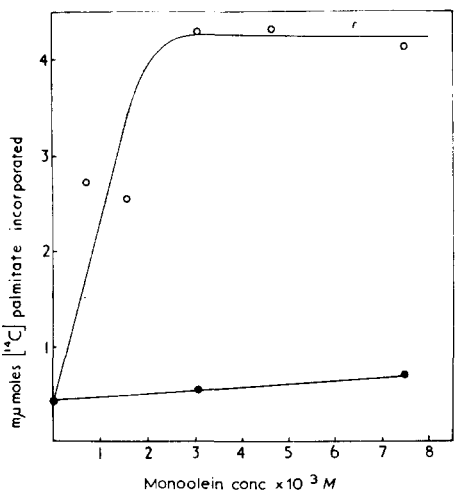

Fig. 1. The effect of varying the concentration of monoolein. The assay system was that described in Table II, except that the concentration of monoolein was changed as indicated. ○—○, complete system; ●—●, ATP and CoA omitted.

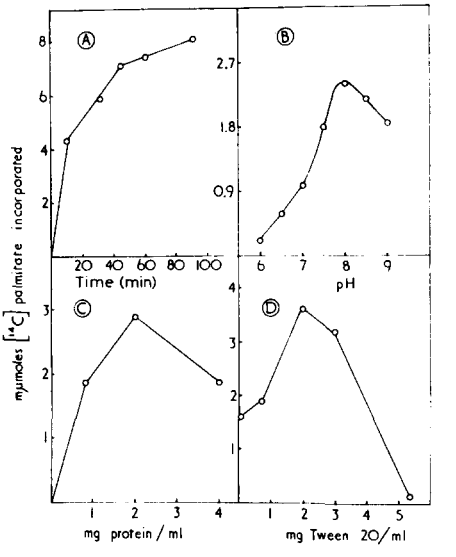

Fig. 2. The effect of time of incubation, final pH value, concentration of protein and of Tween 20. The assay system was that described in Table II, except that the time of incubation (A), final pH value of the assay system (B), concentration of mitochondrial protein (C), and of Tween 20 (D) were varied as indicated.

In Table III the results are given of experiments where cofactors were omitted from or added to the incubation medium.

The omission of ATP from the system caused a marked reduction in the amount of radioactive fatty acid incorporated when either monoglyceride preparation was used. A similar effect was observed when CoA, or CoA and ATP were omitted. The inhibitory effect of Mg^{2+} on the biosynthesis of glycerides using monoglyceride as the acceptor molecule is also shown in Table III. An inhibitory effect of low concentrations of $MgCl_2$ was also observed while studying the biosynthesis of glycerides in subcellular fractions of rabbit small-intestinal mucosa[19,20]. Omitting monoolein from the system, but with ATP and CoA present, caused a marked reduction in the amount of palmitate incorporated.

The requirement for ATP and CoA suggested that palmityl CoA is an intermediate

in the incorporation of free palmitic acid into higher glycerides. The replacement of ATP, CoA and [^{14}C]palmitate by [^{14}C]palmityl CoA is shown in Table IV.

It is of interest that the further addition of ATP did not bring about an increase in incorporation above that observed with palmityl CoA alone. If the phosphorylation of monoglyceride (to give a lyso-phosphatidic acid) was an essential step in the formation of higher glycerides from monoglyceride, a requirement for ATP should have been observed.

Time course

The incorporation of [^{14}C]palmitate as a function of the time of incubation is shown in Fig. 2a. The enzyme preparation used for this experiment had been stored at −10° for three weeks, and had been frozen and thawed twice during that period.

Effect of varying the pH

A study was made of the effect of the final pH value of the incubation system

TABLE III

COFACTOR REQUIREMENT

The assay system was the same as that given in Table II.

Expt.	*Additions*	*mμmoles [^{14}C]palmitate incorporated*
1	Complete system (with α-monoolein)	6.57
	Omit ATP	1.21
	Omit CoA	4.69
	Omit ATP and CoA	1.04
	Omit monoolein	1.95
2	Complete system (with mixed monoolein)	7.42
	Omit ATP	3.73
	Omit CoA	4.55
	Omit monoolein	1.56
	Complete system + 6.7 mM $MgCl_2$	6.22
3	Complete system (with mixed monoolein)	10.45
	Omit monoolein	4.50
	Replace monoolein by glycerol 3.3 mM	4.45
	Replace monoolein by 3.3 mM α-glycerophosphate	8.25
	Replace monoolein by 3.3 mM α,β-diglyceride	5.65

TABLE IV

THE EFFECT OF [^{14}C]PALMITYL CoA

The assay system contained 1 ml of partially purified mitochondrial preparation (5.8 mg protein) in 0.066 M phosphate buffer (pH 7.4) and 20 μmoles of KF. The other additions were made as indicated in the table and the final volume was made up to 3.0 ml with deionised water. The tubes were incubated for 30 min at 37°.

Additions (μmoles added)	*mμmoles palmitate incorporated*
[^{14}C]Palmityl CoA (0.5) + monoolein (10)	13.4
[^{14}C]Palmityl CoA (0.5) + monoolein (10) + ATP (20)	12.8
Hydrolysed [^{14}C]palmityl CoA (0.5) + monoolein (10)	2.1
[^{14}C]Palmityl CoA (0.5)	2.3
[^{14}C]Palmityl CoA (0.5) + monoolein (10) + boiled enzyme	0.2

on the incorporation of palmitate using monoolein as the acceptor molecule. The curve shown in Fig. 2b has been corrected for the incorporation due to lipase action and the values given represent the energy-dependent incorporation only. The pH optimum was found to be 8.0 whereas the pH optimum of the lipase-catalysed exchange reaction lies near 5.0 (see ref. 20).

Effect of increasing the protein concentration

When monoolein and cofactors were incubated with varying amounts of mitochondrial sediment the result shown in Fig. 2c was obtained. The optimal protein concentration was found to be 1.5–2.5 mg/ml for the assay system used. Higher concentrations of protein gave slightly lower incorporations. A similar effect was observed with the preparations from rabbit small intestine[19,20].

Effect of Tween 20

Low concentrations of Tween 20 activated the system, with an optimum concentration of 2 mg/ml. Higher concentrations of Tween 20 reversed the stimulation. At a concentration of 3.5 mg/ml, the amount of palmitate incorporated was again similar to that observed in the absence of Tween 20. The values shown in Fig. 2d were also corrected for lipase activity to give only the energy-dependent incorporation of [^{14}C]palmitate.

Stability of the system

There was little or no loss of activity when the mitochondrial sediment was stored at −20° for several days, and only a 10–20 % loss of activity when kept at this temperature for three weeks.

Esterification of various monoglycerides

Different monoglycerides were tested for their ability to act as acceptors for

TABLE V

The assay system was the same as that in Table II, except that monoglycerides (all made up in Tween 20 to give a final concentration of 2 mg/ml) were added as indicated (10 μmoles ester per test).

Expt.	*Acceptor*	*Structural features*	*mμmoles of ^{14}C-labelled acid incorporated*
1	Monoacetin	C_2 Saturated	0.1
	Monoheptylin	C_7 Saturated	2.2
	Monocaprylin	C_8 Saturated	3.0
	Monocaprin	C_{10} Saturated	6.7
	Monoundecylin	C_{11} Saturated	7.0
	α-Monopalmitin	C_{16} Saturated	3.4
	α-Monostearin	C_{18} Saturated	1.8
2	α-Monostearin	C_{18} Saturated	4.8
	α-Monoolein	C_{18} One double bond	8.2
	Monolinolein	C_{18} Two double bonds	4.9
	Monoricinolein	C_{18} One double bond and one hydroxy group	5.00

activated [^{14}C]palmitate. The saturated substrates with optimal activity were those with a chain length of C_{10} and C_{11} (see Table V). Monoolein had a similar activity to monocaprin or monoundecylin. However, the degree of emulsification of the substrates having saturated fatty acids may be the most important factor in such a comparison. It is significant that substrates with shorter-chain fatty acids (C_2 to C_8) although more water-soluble and far easier to emulsify in water, were much poorer acceptor molecules than those with longer-chain fatty acids.

A comparison was also made between monoolein, monostearin, monoricinolein and monolinolein (see Table V). Monoolein was the most active of these monoglycerides, the other three having about the same activity.

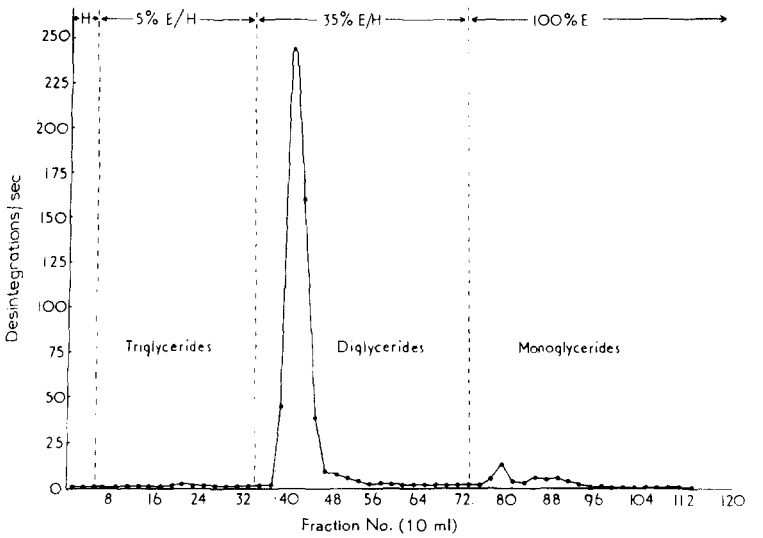

Fig. 3. Separation of reaction products. The separation of the reaction products was done as described by BARRON AND HANAHAN[25] using 30 g of Mallinkrodt silicic acid. The sample was applied in *n*-hexane and for elution 5% ethyl ether in *n*-hexane, 35% ethyl ether in *n*-hexane and ethyl ether were used.

Separation of radioactive glycerides

The radioactive products from several experiments using the mitochondrial sediment as source of enzyme and monoolein as acceptor, were separated on silicic acid by the method of BARRON AND HANAHAN[25]. The predominant product was diglyceride. The actual percentages of the three glycerides were: triglycerides, 1.87; diglycerides, 88.80; monoglycerides, 9.33% of the total radioactivity.

DISCUSSION

The cell fractionation showed that the enzyme system catalysing the esterification of monoglycerides by [^{14}C]palmitate, with diglycerides as the main product, has a widespread distribution in the mucosal cell of rat small intestine and one can conclude that it exists in two forms. A soluble one in the particulate-free supernatant, and a particulate one. The latter form of the enzyme may be regarded as part of the mitochondrial structure (or of the endoplasmic reticulum). A different intracellular dis-

tribution of monoglyceride transacylase was reported for the mucosa of cat small intestine[34]. In that tissue, the microsomal fraction contained most of esterifying activity when monoglyceride was used as glyceride–glycerol precursor. However, there were significant changes in the method of preparing cell fractions from cat gut which might partly account for the differences.

The mitochondrial sediment of rat small-intestinal mucosa had a strong requirement for ATP and CoA, but not for Mg^{2+}. The requirement for ATP and CoA indicated the formation of palmityl CoA prior to transfer of the fatty acid to the monoglyceride and it was shown to be so by replacing ATP, CoA and the free fatty acid by palmityl CoA.

There was a small but significant incorporation of free palmitate in the absence of added ATP and CoA which may have been due to the presence of small amounts of these compounds in the preparation since none of the preparations was dialysed prior to incubation. Secondly, a lipase-catalysed exchange reaction may be responsible for the incorporation of palmitate in the absence of a source of energy. A previous communication has dealt with this aspect[21]. It may be added that the lipase-catalysed incorporation was partially inhibited by 0.66 mg of Tween 20/ml. This concentration of Tween 20 activated the energy-dependent incorporation considerably (see Fig. IId).

The requirement for monoolein in the incubation medium shows that the activated fatty acid does not react to any appreciable extent with acceptors already present in the enzyme preparation. The fact that free glycerol did not act as an acceptor of the activated palmitate indicates that the monoglyceride is unlikely to undergo hydrolysis before esterification, a result in agreement with those of JOHNSTON AND BROWN[35] who used doubly labelled monopalmitin. Since α-glycerophosphate, but not free glycerol was found to be a glyceride–glycerol precursor using the extracted mitochondrial sediment, it may be assumed that glycerokinase activity was absent from this preparation. This enzyme was recently found to occur in the particulate-free supernatant of mucosal homogenates from cat small intestine[36].

The results obtained by incubating palmityl CoA alone or palmityl CoA plus ATP (the other additions being the same (see Table IV)) indicate that lyso-phosphatidic acid can be excluded as an intermediate in the pathway from monoglycerides to di- and triglycerides. The same conclusion was reached from radioisotope dilution experiments with phosphatidic acid using either mitochondrial preparations from rabbit small intestine[19] or particulate-free supernatant from pig kidney[21] as source of enzyme for the esterification of monoglycerides. These results are also in agreement with the data of SENIOR AND ISSELBACHER who used glycerol-labelled monopalmitin and palmityl CoA and ATP[22]. PIERINGER AND HOKIN recently described the formation of lysophosphatidic acid from monoglyceride and ATP using brain preparations[37]. Although this reaction may possibly play a role in the formation of phosphatidic acid in brain tissue, evidence to date indicates that it has no importance for the biogenesis of glycerides in small-intestinal mucosa.

The predominance of diglyceride in the reaction products and also the slow acylation of α,β-diglyceride (as compared with the acylation of monoglyceride) points to either an inactivation of the diglyceride transacylase during the preparation of the mitochondrial sediment or to a better emulsification of the monoglyceride in water, or to different cofactor requirements for the monoglyceride and diglyceride transacylase respectively.

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