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THE INCORPORATION OF CHOLESTEROL INTO INNER MITOCHONDRIAL MEMBRANES AND ITS EFFECT ON LIPID PHASE TRANSITION

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Summary

Incubations of rat liver inner mitochondrial membranes with liposomes prepared from 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and cholesterol resulted in a considerable enrichment of the cholesterol composition of these membranes. This enrichment is not accompanied by an alteration in the membrane phospholipid content or fatty acid composition. The exogenous cholesterol appears to be integrated into the membrane structure because it has effects consistent with the known properties of this sterol in other natural and artificial membrane systems.

Differential scanning calorimetry on both intact membranes and extracted lipids showed that as the ratio of cholesterol to phospholipid was increased, the endotherm corresponding to the lipid phase transition was reduced. Freeze-fracture electron microscopy of the native membranes showed that intramembranous particles are randomly distributed above the phase transition temperature. Below this temperature large smooth areas, believed to correspond to lipid in the gel state from which proteins have been excluded, can be observed. In the presence of high concentrations of cholesterol the fracture faces observed below the lipid transition temperature show no regions of phase segregation, an observation consistent with previous studies using pure lipids where cholesterol was observed to prevent the lipid undergoing a cooperative phase transition.

The results are discussed in terms of the observed low concentrations of

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Abbreviation: DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine.

cholesterol in normal liver inner mitochondrial membranes and the distribution of cholesterol within the liver cells.

Introduction

It is well established that the cholesterol content of mammalian cell membranes is, under normal conditions, consistent for a specific membrane but varies from one membrane to another [1]. An example of this is found in rat liver cells, where the plasma membrane is rich in cholesterol, but the inner mitochondrial membrane has only traces of cholesterol.

The mechanism by which the cholesterol content of membranes is regulated is not understood. Cholesterol can exchange between subcellular membrane fractions in vitro [1] and it is now suggested that the transport of cholesterol from sites of cholesterol synthesis to some subcellular membranes may be facilitated by cytosolic proteins [2]. In the case of the mitochondria this shuttle mechanism may be essential for the synthesis of steroid hormones in steroidogenic tissues [3]. We have therefore considered the possibility that the cholesterol content of membranes may be determined (a) by the nature of the other membrane components or (b) by the availability of cholesterol to a specific membrane.

The effects of cholesterol on the properties of membrane phospholipids have been studied extensively using a variety of physical techniques. Cholesterol lowers the transition temperature of phospholipids observed with differential scanning calorimetry and the endotherm is reduced with increasing amounts of cholesterol [4,5]. Cholesterol has been shown to decrease the fluidity of membrane phospholipids in the liquid crystalline state [4].

In this work we have employed a technique developed to increase the content of erythrocyte membranes [6], using cholesterol-rich liposomes, to establish whether inner mitochondrial membranes can accept large quantities of exogenous cholesterol or whether this capability is limited by the composition of the membrane.

With differential scanning calorimetry and freeze-fracture techniques we have shown changes in membrane phase transitions, which are consistent with the incorporation of this cholesterol into the membrane structure.

Materials and Methods

Chemicals. Cholesterol (chromatography grade), cholestane and Lubrol WX were obtained from Sigma Chemical Co., London, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) from Fluka Ltd, Basel, Switzerland.

Preparation of cholesterol-rich liposomes. Cholesterol and DPPC (molar ratio, 2 : 1) were colyophilized from benzene : methanol (95 : 5) and dispersed in distilled water at a concentration of 6 g/l in 3 ml aliquots. The dispersion was then sonicated in a sealed vessel under N_2 at 30° C for 12 min using an M.S.E. 100 W sonicator fitted with a titanium probe. To remove undispersed lipid and probe debris the solution was centrifuged at 30 000 × *g* for 30 min in an M.S.E. SS50 centrifuge (10 × 10 Rotor) at 8° C.

Preparation of inner mitochondrial membranes. Liver mitochondria were prepared from male Sprague-Dawley rats weighing 250—300 g and the inner mitochondrial matrix fraction was obtained by the method of Schnaitman and Greenawalt [7]. The purity of the membrane was determined by assay of monoamine oxidase activity [8] as an outer mitochondrial membrane marker and cytochrome *c* reductase for the inner mitochondrial membrane after solubilization with Lubrol [7].

Enrichment of inner mitochondrial membranes with cholesterol. Cholesterol-rich liposomes (1 or 4 mg phospholipid/ml medium) were incubated with inner mitochondrial membranes (2.5 mg protein/ml in a medium containing 220 mM mannitol/70 mM sucrose/2 mM Hepes (pH 7.4), 0.5 g/l bovine serum albumin, benzylpenicillin (100 units/ml) and streptomycin sulphate (100 μg/ml). Incubations were carried out in a gently shaking water bath at 20° C under N_2.

At various times, aliquots were removed and diluted with 2 vols. incubation medium (4° C) and centrifuged at 25 000 × *g* for 30 min (MSE SS50 centrifuge, 10 × 10 ml rotor). The pellet was taken up in 0.52 M sucrose/2 mM Hepes, pH 7.4, and layered over 0.68 M sucrose/2 mM Hepes, pH 7.4, and beneath 100 mM KCl/2 mM Hepes, pH 7.4. The layers were then centrifuged at 100 000 × *g* for 90 min (MSE SS50 centrifuge, 3 × 25 ml rotor) at 4° C. The inner mitochondrial membrane was recovered as a pellet at the bottom of the tube and the liposomes banded at the interface of the 0.52 M sucrose and 100 mM KCl solutions.

Differential scanning calorimetry. To the spherical inner membrane vesicles in 40 mosM incubation medium was added ethylene glycol to 50% v/v [9]. The membranes were pelleted at 15 000 × *g* for 30 min at 4° C and transferred to a 100 μl matched-weight stainless steel specimen capsule and sealed. A reference sample contained the same weight of Sephadex G-200 (20 g/l swollen in 40 mosM incubation medium containing 50% v/v ethylene glycol). The membrane samples were scanned in a Perkin-Elmer differential scanning calorimeter 2. (Perkin-Elmer Ltd, Beaconsfield, Bucks, U.K.) at a heating and cooling rate of 5 or 2.5 K/min and at a sensitivity of 0.2 mcal/s. Extracted membrane lipids were scanned at a sensitivity of 1 or 0.5 mcal/s. Cyclohexane and indium were used to calibrate the instrument. Each sample was run at least twice.

Analytical techniques. Lipids were extracted from membranes and liposomes [10] and lipid phosphorus determined by a colorimetric assay [11]. Cholesterol was extracted by the method of Rose and Oklander [12] after saponification of the phospholipids. It was quantified by GLC using a Pye 204 gas chromatograph (Pye-Unicam Ltd, Cambridge, U.K.) fitted with a 5 ft glass column containing 3% QF1 on diatomite CLQ (J.J. Chromatograph, Kings Lynn, Norfolk, U.K.) at 220° C and a N_2 flow rate of 60 ml/min. Cholestane was used as an internal standard. The fatty acid content of the membranes was analysed as described previously [13]. Protein concentrations were determined by the method of Lowry et al. [14].

Freeze-fracture electron microscopy. For freeze-fracture studies, inner mitochondrial membranes were incubated in 40 mosM incubation medium containing 10 mM Tris/HCl buffer, pH 7.4, to which glycerol was then slowly added to a final concentration of 30% v/v and equilibrated for 5 min at room temperature.

The samples were pelleted at room temperature and transferred to gold-nickel specimen holders which were then equilibrated at the required temperature for 30 min before being rapidly quenched in a slurry of solid and liquid N_2. Fracturing and platinum-carbon replication were carried out at −112°C and $2 \cdot 10^{-6}$ Torr using a Polaron freeze-fracture module (Polaron Ltd., Watford, Herts, U.K.). Micrographs were obtained with a Phillips EM301G electron microscope operated at 80 kV.

Results

Enrichment of mitochondrial inner membrane with cholesterol

The inner membrane of rat liver mitochondria was found to have a cholesterol : phospholipid molar ratio of less than 1 : 20, but on incubation with liposomes prepared from cholesterol and DPPC (molar ratio 2 : 1) the cholesterol content of these membranes increased with both longer periods of incubation and higher concentrations of the cholesterol-rich liposomes (Fig. 1). Molar ratios of 1 : 2.5 cholesterol to phospholipid and higher were achieved with long incubations, a greater than 10-fold increase over normal values.

Further analysis of both protein and phospholipid contents of the membranes, both before and after the incubations, revealed differences of less than 5%. No significant change in the fatty acid composition of the mitochondrial lipids was observed, indicating that DPPC was not exchanged into the inner mitochondrial membrane from the liposomes, nor did any net transfer of DPPC occur. The only change in lipid composition was therefore due to the net transfer of cholesterol from the liposomes to the mitochondrial membrane.

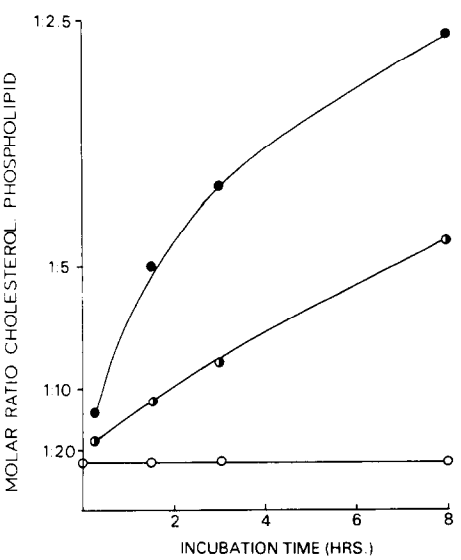


Fig. 1. The rate of incorporation of cholesterol into inner mitochondrial membranes. The membranes were incubated at 20°C in the absence of liposomes (○); or in the presence of liposomes (molar ratio 2 : 1, cholesterol : DPPC) at a concentration of 1 mg DPPC/ml (◑); or 4 mg DPPC/ml (●). Incubation conditions are given in the text.

The inner mitochondrial membrane therefore appears to have the ability to accept large quantities of cholesterol despite its normally small content of this sterol. However, it cannot be concluded that the cholesterol had entered the phospholipid matrix and was not simply adhering to the surface of the membrane. The studies described below show that it was truly interpolated into the bilayer.

Differential scanning calorimetry

Lipid phase transitions were studied in normal and cholesterol-enriched membranes (Fig. 2). In non-incubated inner mitochondrial membranes and in those inducated in a liposome-free medium for 8 h, an endotherm is observed between 275 and 290 K. In membranes which have been incubated with cholesterol-rich liposomes to give a final C/P (cholesterol/phospholipid) ratio of 1 : 8 only small reductions in the endotherm were noticed. However at higher C/P ratios (1 : 2.5) the endotherm was considerably reduced.

Similar studies were performed on the lipid extracts of inner mitochondrial membranes to which increasing quantities of cholesterol had been added. Here again the endotherm is lowered as the molar ratio of cholesterol to phospholipids is increased (Fig. 3).

The transferred cholesterol affects the endotherm of the membrane lipids in a manner consistent with it being fully incorporated into the membrane structure.

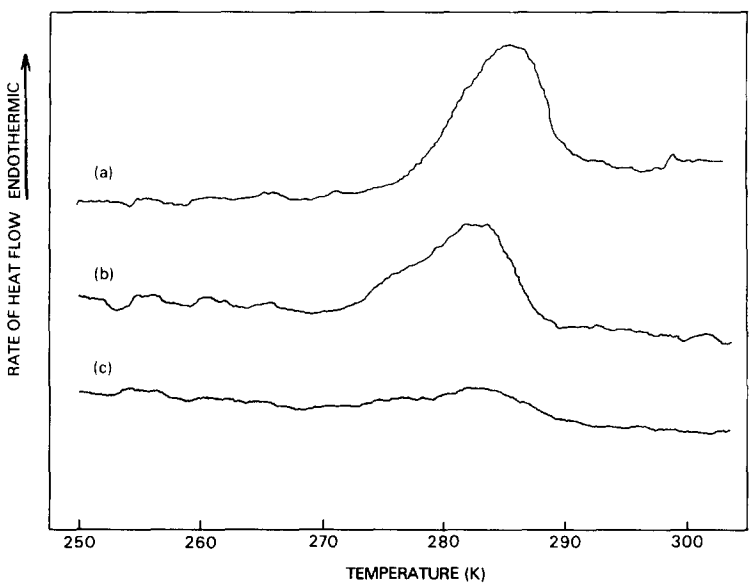


Fig. 2. The effect of incorporated cholesterol on the phase transition of the inner mitochondrial membrane shown by differential scanning calorimetry. The scans show the lipid phase transition of (a) the native membrane (C : P ratio, 1 : 22); (b) the membrane with a C : P ratio of 1 : 8; and (c) with a cholesterol : phospholipid ratio of 1 : 2.5.

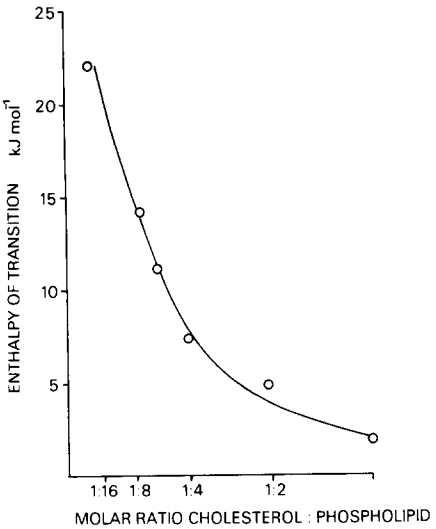


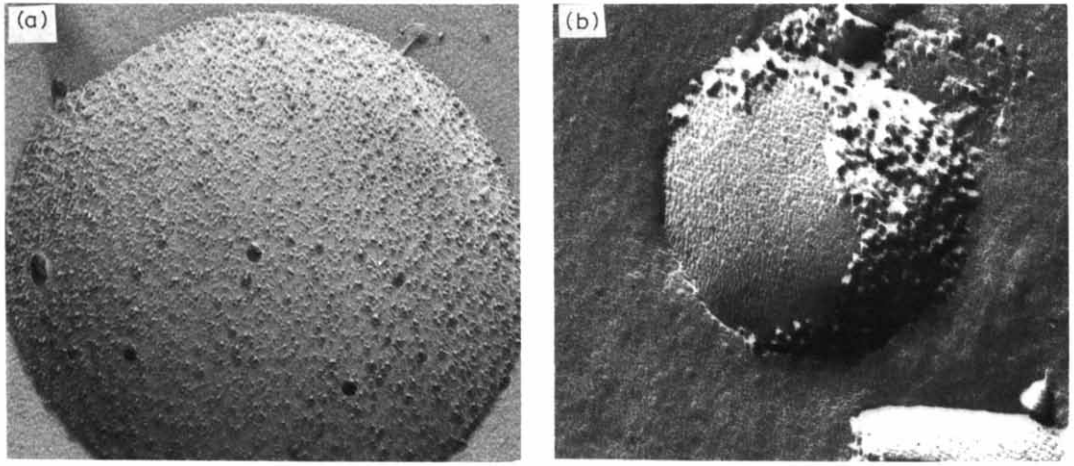
Fig. 3. The effect of cholesterol on the enthalpy of transition of the extracted inner mitochondrial lipids as shown by differential scanning calorimetry. Cholesterol was added to the extracted lipids to give the required C : P ratio.

Freeze-fracture studies

Freeze-fracture of the native inner mitochondrial membranes was carried out both above and below the endothermic transition temperature revealed by DSC. Above this temperature the fracture faces show intramembranous particles randomly distributed in the lateral plane of the membrane (Fig. 4a). In fracture faces produced below the transition temperature large smooth areas devoid of intramembranous particles were observed (Fig. 4b) and are believed to represent areas of lipid in the gel state [9]. Further samples were etched from membranes enriched with cholesterol. In membranes with a C/P ratio of 1 : 18 (Fig. 4c) large smooth areas were still observed in fracture faces prepared below the transition temperature. However, as the cholesterol content of the membranes was increased, smaller smooth areas were observed (Fig. 4d) until at C/P ratios of 1 : 3 and above no phase separation could be detected (Fig. 4e). After incubation at 37°C (Fig. 4b—e), the inner mitochondrial membrane vesicles lose the ability, shown by fresh preparations (Fig. 4a), to swell in hypotonic media. Consequently, smaller fracture faces are observed and comparisons of particle densities are not meaningful. The results are therefore compatible with those obtained by DSC where the transition endotherm between gel and liquid crystalline state was largely eliminated by the presence of cholesterol, a characteristic effect of the sterol when present in a membrane.

Discussions

Mitochondrial membranes have the lowest cholesterol content of all subcellular membranes in liver cells and this is especially true of the inner membrane. A number of explanations may by advanced for this. The composition of the phospholipid classes is unique, with the presence of large amounts of



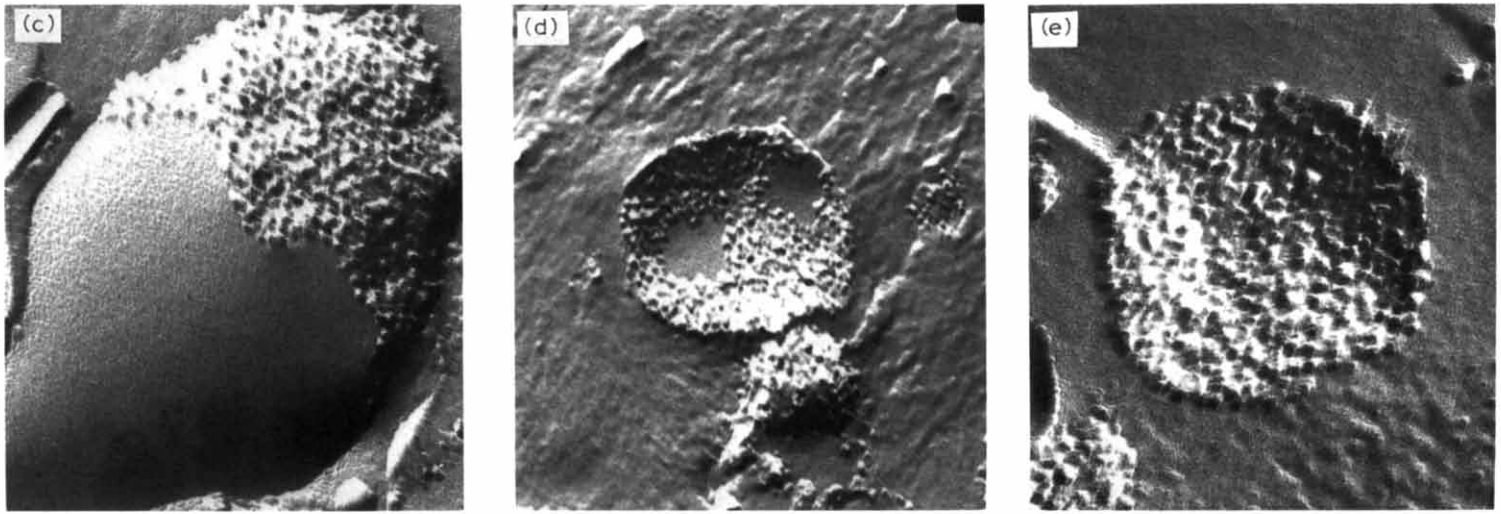


Fig. 4. Freeze-fracture micrographs of native inner mitochondrial membranes produced (a) above and (b) below the transition temperature. Membranes with a cholesterol : phospholipid ratio of (c) 1 : 18 (d) 1 : 6 and (e) 1 : 3 were also observed below the transition temperature. The chosen temperatures from which samples were quenched were 20°C and −15°C. The magnification for a and d was ×51 000 and for b, c and e ×66 600.

cardiolipin and very small quantities of sphingomyelin [1]. High concentrations of sphingomyelin have been associated with high cholesterol/phospholipid ratios in cell membranes [15] and a special association of the two lipids has been proposed [16]. Another possibility is that that the rate of transport of cholesterol from its site of synthesis to the outer mitochondrial membrane, which normally has a higher content of cholesterol than the inner membrane, is a controlling factor.

This rate of transfer has been shown to be accelerated in vitro by proteins present in the cytosol [2], but in vivo this may still limit the accumulation of cholesterol in mitochondrial membranes. The intermembranous space between the mitochondrial membranes is another barrier to the acquisition of cholesterol by the inner membrane.

Incubation of whole mitochondria with plasma brought about an enrichment of both inner and outer mitochondrial membranes with cholesterol, but not exceeding a 2-fold increase over normal levels [17]. This enrichment was sufficient to modify the swelling amplitude of the mitochondria. Modest increases in liver mitochondrial cholesterol content were obtained in vivo following injection of rats with thyroxine [18].

Evidence is now presented that liver inner mitochondrial membranes are capable of incorporating large quantities of cholesterol from a cholesterol-rich source. The final concentration is dependent on the concentration of the cholesterol-bearing liposomes and the duration of their incubation with mitochondria. The C/P ratios reached values comparable to those normally observed in liver cell plasma membranes and lysosomes and greater than those typical of the endoplasmic reticulum [1]. This strongly suggests that the dearth of cholesterol in the inner mitochondrial membrane is not due to the peculiar chemical composition of the membrane but to the limited transfer of cholesterol from the endoplasmic reticulum.

The actual concentration gradient, in terms of C/P ratio, between endoplasmic reticulum and the outer mitochondrial membrane is not large and it would therefore appear that the transport of cholesterol by cytosolic proteins is sufficient to permit a near equilibrium situation between these membranes. The lower concentration of cholesterol in the inner mitochondrial membrane may arise from a limitation in its transfer across the intermembranous space. In adrenal mitochondria, which are relatively rich in cholesterol, the transport of cholesterol to the inner mitochondrial membrane, where it is converted to pregnenolone, may be mediated by a carrier protein [19]. Indeed inhibition of the enzyme responsible for this conversion, desmolase, leads to a large accumulation of cholesterol in the mitochondria, as does treatment with the hormone corticotrophin [20].

The concentration of cholesterol in plasma membranes may arise from entirely different processes, either through the accumulation of cholesterol from extracellular pools or a more rapid metabolic turnover of glycerophospholipids compared with cholesterol or sphingolipids, all of which are synthesized in the endoplasmic reticulum. It has also been suggested that there is an asymmetry of cholesterol across the membranes [21] which may lead to a lower C/P ratio on the cytosolic face of the plasma membrane, lessening the gradient between plasma membranes and the other internal membranes of the cell. The

asymmetry now appears to be in doubt [22].

We have also provided evidence that cholesterol was introduced into the inner mitochondrial membrane without a change in the composition of other membrane lipids and was fully incorporated into the membrane structure. There is abundant evidence that cholesterol lowers the transition temperature of phospholipids and abolishes the endotherm [23] when present in large quantities (C/P ratio, 1 : 1). It also affects the permeability properties [24] and enzyme activities of membranes [25,26], including effects on the cytochrome P450 system in adrenal mitochondria [27] and other mitochondrial enzymes [17].

The introduction of large quantities of cholesterol into inner mitochondrial membranes will not only increase the total lipid content of the membranes, but also modify their physical properties. This is clearly demonstrated in results described above where the transition temperature of lipids in the native membranes and lipid extracts is essentially eliminated by the presence of cholesterol. The remaining small endotherm in the extracted lipids (C : P ratio, 1 : 1) may be due to the presence of 'neutral' lipids, e.g. diglycerides and triglycerides in the mitochondrial extract.

Intramembranous particules are thought to be excluded from areas in the mitochondrial membrane where lipids are in the gel state and aggregate, resulting in the appearance of smooth areas in electron micrographs of freeze-fracture faces [9]. Cholesterol abolishes the transition between the gel and liquid crystalline states and introduces a state of 'intermediate' fluidity [4]. However in freeze-fracture studies with (Ca^{2+} + Mg^{2+})-dependent ATPase from sarcoplasmic reticulum incorporated into liposomes prepared from synthetic saturated phospholipids [28] the inclusion of 20 mol% cholesterol gave rise to linear arrays of protein molecules, which were not observed in this current work. Our results are again consistent with the incorporation of cholesterol into the bilayer and not the result of adhesion of extraneous cholesterol to the membrane. We conclude that further study of the distribution of cholesterol in cells should be concentrated on rate limiting steps for cholesterol transport from sites of synthesis or from external sources rather than the effect of membrane lipid composition on the efficacy of a membrane as a cholesterol acceptor.

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