Rates of removal and degradation of chylomicron remnants by isolated perfused rat liver

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Abstract
Chylomicron remnants are removed intact by isolated perfused rat livers and their lipid components are metabolized by the liver (Biochim. Biophys. Acta 488: 464, 1977.). The present study provides quantitative information regarding these processes. When the lipoprotein concentration of the perfusate was constant, the removal of chylomicron remnants increased linearly for 17 min. The rate of remnant removal was a hyperbolic function of the perfusate's remnant concentration. The removal rate had a $V_{\text{max}}$ of 28 µg cholesterol per g liver per min and an apparent $K_m$ of 64 µg cholesterol per ml perfusate. Feeding the liver donors a diet containing 1% cholesterol or 4% cholesterol and 1% cholic acid failed to alter the hepatic removal rate. The cholesteryl ester removed from the remnants was hydrolyzed at a rate that was a small fraction of the removal rate (about 0.5% of removed cholesteryl ester per min). The rate of cholesteryl ester hydrolysis did not appear to approach saturation in the range studied. Studies of the lysosomal cholesteryl ester hydrolase suggested that this enzyme was not responsible for limiting the initial rate of hydrolysis, raising the possibility that the degradation rate is determined by the movement of the removed remnant to the site of hydrolysis.

Supplementary key words cholesterol metabolism · cholesterol esterase · cholesterol feeding

METHODS

Since the description of the chylomicron remnant by Redgrave (1), considerable information has accumulated concerning the physical and biochemical properties of these particles (1–4). Their role in overall lipoprotein homeostasis is just beginning to emerge. Two aspects are of particular interest. First, since chylomicron remnants are the same size as VLDL particles it is possible that they may contribute significantly to the circulating VLDL pool. Although Grundy and Mok (5) did not find evidence for this in man, Ross and Zilversmit (6) found that, in cholesteryl-fed rabbits, remnants were the primary constituents of the circulating VLDL pool. Second, once removed by liver, remnants make a substantial contribution to the hepatic cholesterol content. Several investigators have been able to correlate the hepatic cholesterol content with rates of hepatic cholesterol synthesis (7, 8). However, the correlation is not always precise, especially with respect to the time course of the inhibition. This may be because the rate and manner in which remnants enter the hepatic cholesterol pool is not completely understood.

Before being able to answer questions such as these, better quantitative data concerning the hepatic metabolism of chylomicron remnants is required. The isolated perfused liver is a good system for obtaining this information. Chylomicron remnants have been found to be removed as a unit by isolated liver (9). Moreover, the rates of catabolism of cholesteryl ester from remnants in vivo and by isolated liver are similar and the suppression of cholesterol synthesis, which occurs after cholesterol ingestion in vivo, also occurs after the removal of chylomicron remnants by isolated livers (9). The purpose of the present study was to obtain quantitative data regarding the rate of removal and degradation of these particles by isolated liver, and to attempt to identify the limiting steps of the process.

Animals

All rats were males of the Sprague-Dawley strain. They were housed in a windowless room, illuminated 7 AM to 7 PM, and fed a standard chow or experimental diet. The experimental diet was prepared by adding cholesterol dissolved in ether, and, in some instances, cholic acid dissolved in alcohol, to ground standard rat chow. The solvent was then evaporated under nitrogen for at least 24 hr. Liver donors weighed 160–200 g and were purchased 2 weeks before use. For experiments with cholesterol-fed animals, rats were randomly divided into two groups at the time of acquisition (60 g) and placed in separate cages. They were fed either the cholesterol-rich diet or ground chow without sterol. The animals fed the 4% cholesterol–

Abbreviations: VLDL, very low density lipoproteins; LDL, low density lipoproteins.
1% cholic acid diet gained less weight than the controls (125 ± 3 g for control vs. 105 ± 4 g for cholesterol-fed animals). Lymph donors weighed 300–400 g and retired breeders were used to prepare chylomicron remnants.

**Materials**

Cholesterol, cholesteryl oleate, cholic acid, and phenolphthalein mono-β-glucuronic acid were purchased from Sigma (St. Louis, MO). Radioisotopes were purchased from New England Nuclear (Boston, MA). Cholesterol and cholesteryl ester were purified by thin-layer chromatography (silica gel H plates developed in hexane–ether–acetic acid 85:15:2). Egg lecithin was purchased from Grand Island Biological Co. (Grand Island, NY).

**Lipoprotein preparation**

Chylomicrons containing radiolabeled cholesterol were obtained as previously described (9). In early experiments remnants were prepared in eviscerated rats by the method of Bezeman-Thatcher, Otway, and Robinson (10). After the rats were anesthetized with pentobarbitol, the aorta was cannulated cephalad to the celiac artery. The esophagus was ligated; the inferior vena cava was ligated cephalad to the renal vein and was cannulated through the hepatic vein which was ligated around the cannula. One ml of chylomicrons containing no more than 2.5 mg of cholesterol was injected through the inferior vena cava and this was followed by a continuous infusion of 5% dextrose in sterile water (1 ml/hr). After 1 hr the animal was exsanguinated through the aortic cannula while 0.9% NaCl was infused through the vena cava. In later experiments, the eviscerated animals used were prepared, as described by Redgrave (1), by ligating the celiac, superior, and inferior mesenteric arteries and the portal vein. The lipoprotein was injected through the femoral vein and allowed to circulate for 3 hr. No more than 8 mg of cholesterol was injected. Results with the two methods were identical as long as the restrictions on amount of lipoprotein injected were followed. Remnants so prepared had a triglyceride to total cholesterol ratio (wt:wt) of 5.5 ± 0.6. This represents a somewhat greater depletion than with the in vitro method (9) of producing these particles. After a 1-hr incubation at room temperature, the red blood cells were removed, washed with 0.9% NaCl, and the plasma and saline wash were centrifuged at 1.6 × 10^4 g for 120 min in an SW41 rotor. The lipoproteins were removed by tube slicer and resuspended in 0.9% NaCl through a 25 gauge needle.

**Experimental design. Removal and hydrolysis experiments**

Liver perfusion was performed as described previously (11). The perfusion was established with 80 ml of a lipoprotein-free perfusate. When flow was properly established and the liver judged viable, the perfusate was changed to one that contained labeled lipoprotein. The volume of this perfusate was 35 ml for high lipoprotein concentrations and was progressively increased to about 50 ml for lower lipoprotein concentrations; it was allowed to recirculate for a period of time, usually 10 min, and then the liver was perfused for an additional 45 min with lipoprotein-free perfusate. After this the liver was flushed with ice-cold 0.9% NaCl and removed. A 1-g portion was homogenized. An internal standard of equal amounts of [³⁵S]cholesterol and [¹⁴C]cholesteryl ester was added to the homogenate, and the lipids were extracted by the method of Folch, Lees, and Sloane Stanley (12).

Perfusate samples were obtained from the tube leading to the liver at the start and at the conclusion of perfusion with lipoprotein-containing perfusate. Red blood cells were removed by centrifugation. An internal standard was added as above. The initial and final concentrations were averaged to estimate the lipoprotein concentration bathing an average hepatocyte. A recirculating system was used because it required considerably less lipoprotein to obtain high lipoprotein concentrations.

In some experiments only removal was measured; for these, the final perfusion with unlabeled perfusate was omitted. In experiments undertaken to determine the effect of cholesterol feeding on removal, sterol-fed and control-fed animals, originally derived from the same group of rats, were perfused with the same lipoprotein-containing perfusate.

The total lipid extracts from both liver and perfusate samples were brought to volume and a test portion was taken from each for liquid scintillation counting in Liquifluor (New England Nuclear). Another portion was applied to a silica gel H thin-layer chromatography plate which was developed in hexane–ether–glacial acetic acid 85:15:2. The areas corresponding to authentic cholesterol and cholesteryl ester were scraped and counted in Liquifluor.

Total uptake and perfusate cholesterol content were calculated from the total ³H present, corrected for recovery of internal standard. The fractions of esterified [³H]cholesterol present in the liver and in the perfusate were calculated. The difference between these multiplied by the amount of cholesteryl ester removed gave the amount of cholesteryl ester hydro-
lyzed. In 45 min, the amount of reesterification or secretion of [3H]cholesterol was insignificant.

**Cholesteryl ester hydrolase**

Lysosomes were prepared by the method of Teng and Kaplan (13). The lysosomal recovery was in the range of 2–6% and enrichment was about 30-fold as determined by specific activity of the lysosomal enzyme phenolphthalein mono-β-glucuronidase.

β-Glucuronidase was measured by the method of Giannetto and DeDuve (14). Phenolphthalein mono-β-glucuronic acid was incubated with lysosomes in 0.075 M acetate buffer, pH 5.2. The reaction was stopped with 0.2 M Na2CO3 and the liberated phenolphthalein was determined spectrophotometrically.

Cholesteryl esterase activity was assayed using either liposomes or remnants as the substrate. Liposome substrate was prepared by the method of Brecher et al. (15). Cholesteryl [14C]oleate (700 μg, 7.5 μCi/μmol) and 60 mg of egg yolk lecithin were dissolved in chloroform–methanol 2:1, dried under N2, and sonicated under N2 for 10 min in 6 ml of 0.1 M NaCl. This was centrifuged at 150,000 g for 30 min and the supernatant was used as substrate.

The reaction mixture consisted of 100 μl of 0.15 M acetate buffer (pH 4.5), 25 μl of lysosome suspension, and different amounts of substrate suspended in 100 μl of 0.1 M NaCl and 0.01 M Tris buffer (pH 7.4). With the artificial substrate the reaction was stopped by the addition of 3.0 ml of benzene–chloroform–methanol 1:0.05:1.2 and 0.6 ml of 0.3 M NaOH (16). After mixing, the layers were separated by centrifugation, an aliquot of the upper layer was removed, and the radioactivity was determined. A protein-free blank was also run.

With remnants as substrate the reaction was terminated by the addition of 20 vol of chloroform–methanol 2:1 and the phases were separated with 0.02% CaCl2. An aliquot of the lipid extract was chromatographed and the percent cholesteryl ester present was determined. The quantity of free cholesterol liberated was calculated. With both substrates the rate of hydrolysis was measured as the percent of the substrate present after incubation time. Activity was calculated as μg of cholesteryl ester hydrolyzed per min per unit of β-glucuronidase activity. This was then converted to grams wet weight from measurement of β-glucuronidase activity in the whole liver homogenate.

**Chemical methods**

Protein was determined by the method of Lowry et al. (17), cholesterol by the method of Leffler (18), and DNA by the method of Wannemacher, Banks, and Wunner (19).

![Fig. 1. Uptake of chylomicron remnants by liver; effect of perfusion time. Isolated livers were perfused with a medium containing Krebs-Ringer buffer, 3% albumin, 22% red blood cells, and chylomicron remnants with a perfusate concentration of 70 μg lipoprotein cholesterol per ml. The perfusate did not recycle and each liver was perfused with the same remnant concentration. After different times, the liver was removed and the quantity of lipoprotein cholesterol in liver determined. Each point is the mean of two determinations.](image_url)

**RESULTS**

**Removal of chylomicrons and remnants by isolated liver**

When livers were perfused with a medium containing a constant concentration of chylomicron remnants (single pass perfusion), the amount of lipoprotein taken up by the liver was a linear function of time (Fig. 1) for at least 17 min.

Next the hepatic removal of lipoprotein was studied as a function of perfusate lipoprotein concentration. For these experiments a 10-min recirculation perfusion with lipoprotein-containing medium was used. The mean perfusate concentration was used for calculations. Even with a single-pass perfusion the mean concentration must be estimated, because the concentration of a substance removed by liver decreases as the blood flows from periportal to pericentral hepatocytes (20). The uptake of chylomicrons by the liver appeared to increase linearly with the perfusate concentration. Incubation of chylomicrons with normal rat plasma for 3 hr did not increase their rate of removal (data not shown). At all perfusate lipoprotein concentrations used, the uptake rate of chylomicron remnants was considerably greater than the rate of uptake of chylomicrons. Moreover, with remnants the uptake process appeared to be saturable and the plot of perfusate concentration against rate of uptake was hyperbolic (Fig. 2). The double reciprocal plot of the removal rate against concentration gave a straight line. From this a $V_{max}$ for uptake of 28 μg
Effect of cholesterol feeding on remnant removal

It has been shown that the rate of removal of LDL by fibroblasts can be depressed by preincubating the fibroblasts with either LDL or cholesterol (21). Similarly, autoregulation of the number of hormone receptors present by exposure to hormones has been demonstrated (22). We have tested whether the same phenomena occur with remnant removal.

When the livers of rats fed a 1% cholesterol-containing diet were perfused with remnants, no significant difference between their uptake rate and that of control livers was noted (Fig. 2). Since such animals do not develop hypercholesterolemia and have only modestly increased hepatic cholesterol content, animals fed 4% cholesterol and 1% cholic acid were also studied. These animals develop hypercholesterolemia and have an increased hepatic cholesterol content and liver size (23). The rate of remnant uptake by these livers appeared to be lower than that of control livers perfused with the same batch of lipoproteins (Fig. 3A). The same relationship was found if the uptake rate was calculated per mg liver protein or per g wet weight. However, when calculated per mg DNA the apparent difference in rates was eliminated (Fig. 3B). These results suggest that the uptake rate per cell was unchanged by cholesterol feeding. Moreover, since the livers of 4% cholesterol-fed animals were larger than those of control animals (11.9 g for cholesterol-fed compared to 8.4 g for controls), their total hepatic lipid uptake was actually greater than that of control rats. Taken together these data suggest that rate of uptake of remnants by liver is not regulated by hepatic cholesterol content.

Hydrolysis of remnant cholesteryl ester

After hepatic removal, the cholesteryl ester from chylomicrons is hydrolyzed to free cholesterol. It has

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Fig. 2. Uptake of chylomicron remnants and chylomicrons by liver; effect of perfusate concentration. Perfusion was performed as in Fig. 1 except the perfusate was allowed to recycle and the concentration of lipoprotein was calculated by averaging the initial and final perfusate concentration. △ Represents livers from control animals perfused with remnants; ○ represents livers from 1% cholesterol-fed animals perfused with remnants; ■ represents livers from control animals perfused with chylomicrons. The curve and kinetic parameters were obtained by least squares regression of a double reciprocal plot of the data from remnant-perfused livers from control-fed animals. The line for chylomicrons was calculated by least squares regression. Each point represents a separate liver.

Fig. 3. Uptake of chylomicron remnants by liver; effect of 4% cholesterol feeding. Perfusion was performed as described in Fig. 2. ■ Represents livers from control fed animals; ○ represents livers from 4% cholesterol and 1% cholic acid-fed animals. The data shown are from a single representative experiment where livers were perfused with the same batch of lipoproteins. In A, results are plotted as µg cholesterol removed per g liver; in B, the data from the same experiment are expressed as µg cholesterol removed per mg hepatic DNA. Each point represents a single liver.
been demonstrated using LDL in fibroblasts that this hydrolysis is an obligatory step in intracellular regulation of cholesterol synthesis and cholesterol reesterification (24). There are, however, few quantitative data concerning the rate of this process in the intact liver. Accordingly, isolated livers were perfused first with a medium containing chylomicron remnants labeled with [3H]cholesterol and then with lipoprotein-free medium. Immediately after the perfusion with lipoprotein-containing medium, there was little difference between the percent of [3H]cholesterol that was esterified in the perfusate and in the liver. During the perfusion without lipoprotein, the total [3H]cholesterol was constant while the percent [3H]cholesterol present as ester in liver decreased progressively (Table 1).

In subsequent experiments the lipoprotein-free perfusion was continued for 45 min. The amount of cholesterol hydrolyzed was calculated by multiplying the difference between the percent [3H]cholesterol as ester in perfusate and liver by the amount of cholesteryl ester removed. A linear relationship between removal and hydrolysis was found (Fig. 4). A hydrolysis rate of about 0.5% of the remnant cholesteryl ester per min was found over the entire range studied. These data provide quantitative confirmation for the concept that the hydrolysis of lipoprotein cholesteryl ester is a slow step relative to removal. Moreover, a double reciprocal plot of the data in Fig. 4 passed through the origin (not shown). This suggests that the rate-determining step of hydrolysis may not be enzymatic. This possibility was further explored by measuring the activity of the lysosomal cholesterol esterase.

Comparison of lysosomal cholesterol esterase activity with hydrolysis by intact liver

The bulk of evidence at present suggests that, in both fibroblasts (24) and liver (25, 26), the lysosomal cholesteryl esterase is the enzyme responsible for hydrolyzing cholesteryl ester of lipoprotein origin. In order to explore the relationship between the rate of hydrolysis of cholesteryl ester from remnants in the intact liver and the enzymes responsible for the process, the kinetics of the lysosomal cholesteryl esterase were determined. Both the liposomal dispersion of cholesteryl ester suggested by Brecher et al. (15) and chylomicron remnants were utilized as substrates (Figs. 5A and B). With the dispersion the \( V_{max} \) was considerably higher than with the lipoprotein particle but the enzyme had a higher affinity (lower \( K_m \)) for the lipoprotein. Since it is not clear which substrate would be most representative of the intact hepatocyte, further comparisons were made utilizing both curves. The dotted lines in Figs. 4 and 5B allow comparison of the cholesterol concentration required to achieve a hydrolysis rate of 0.5 \( \mu g \) per min. From this it is apparent that much less hydrolysis occurs in intact liver than the enzyme data would predict.

**DISCUSSION**

A model for the metabolism of LDL by fibroblasts has been proposed by Brown et al. (27). The sequence of events appears to be a binding of the LDL particle to a specific cell surface receptor, internalization...
(probably by adsorptive endocytosis), fusion of the endocytic vesicle with the lysosomes, and degradation of the lipoprotein by lysosomal enzymes. The particles' lipid components are then released in unesterified form. These lipolytic products cause metabolic changes in the cell and are themselves further metabolized. A similar sequence of events appears to occur in other organs as well.

Studies from several laboratories suggest that liver is an exception because this organ does not appear to be the site of LDL removal (28). In the liver, the chylomicron, via its triglyceride-depleted remnant, rather than LDL appears to be the lipoprotein that has the most profound effect in acutely altering the cholesterol content of the liver and hence the rate of hepatic cholesterol synthesis (29). The present study was undertaken to provide quantitative information about the processes of removal and degradation of the chylomicron remnant by isolated liver.

It was previously shown that the particle appeared to be removed as a unit by the liver (9). In this study it was found that, with a constant remnant concentration in the perfusate, the rate of uptake by liver was directly proportional to the duration of the perfusion. As the remnant concentration was increased, the increment in the rate of removal diminished and the process approached saturation. Similar observations were recently reported by Sherrill and Dietschy (30) who used no red cells and an albumin-free medium in short-term perfusions.

The calculation of kinetic parameters for hepatic removal processes is complicated by the fact that the substrate may not be distributed uniformly in the vascular space and the fact that the concentration of the substrate decreases as the blood passes from periportal to pericentral cells (20). In addition, in our studies, some cycling of perfusate was allowed in order to conserve lipoproteins. To compensate for the change in the substrate concentration during removal, the initial and final perfusate concentrations were averaged. This approach assumes a linear change in substrate concentration during perfusion and across the hepatocyte. Although this represents an obvious oversimplification, the use of other methods (31) requires assumptions that are not necessarily more valid. To test the extreme cases the same calculations were made using either the initial, average, or final perfusate concentrations and $K_m$ values of 8.8, 6.4, and 4.0 mg/100 ml were obtained, respectively. These numbers provide the extreme range within which the $K_m$ could fall.

Similarly, Sherrill and Dietschy (30), using their
simpler perfusion system, obtained a $K_m$ of 4.5 mg 100 ml$^{-1}$ using their initial concentration and of 1.9 mg 100 ml$^{-1}$ using their mean concentration. Somewhat lower values would be expected, because they removed red blood cells and albumin that could serve as barriers to uptake.

To obtain a more precise value for $K_m$ will probably require modification of the multiple isotope, computer-based techniques of Goresky, Bach, and Nadeau (20) and even then intra-animal variation and possible differences in lipoprotein composition will not be eliminated. Nonetheless, the point that emerges from these studies is that the $K_m$ for remnant removal occurs at a low plasma lipoprotein concentration, certainly less than 10 mg of cholesterol per 100 ml, confirming that the liver has a high affinity for these particles. Moreover the $V_{max}$, 28 $\mu$g cholesterol g$^{-1}$min$^{-1}$ in our system and 35 $\mu$g cholesterol g$^{-1}$min$^{-1}$ in that of Sherrill and Dietschy, suggests that under physiologic circumstances this removal process is not likely to approach saturation.

The small amount of chylomicron uptake observed seems to occur by a different mechanism than remnant uptake. Jones reported (32) that when chylomicrons were perfused through a liver they could be found in the macrophages but when heparin was added (and remnants presumably formed) this did not occur. Other evidence also supports the concept that there is no remnant removal by macrophages (33).

The failure to substantially alter the removal rate by cholesterol feeding was somewhat surprising since the amount of binding of LDL to its receptor can be regulated in this way (21). It suggests that remnant removal by liver and LDL removal by fibroblasts may not be precisely analogous. Recent evidence has accumulated that, at least in some species, chylomicron remnants contribute to the circulating VLDL pool during cholesterol feeding. Moreover, the disappearance of injected labeled remnants was slower in cholesterol-fed than in control rabbits (6). If data from the rat is applicable to other species, it appears that this is not the result of a change in the removal mechanisms but is a function of approaching saturation of the normal process. If other factors, such as heredity, diet, or hormones alter the kinetics of this process, remnants could make substantial contributions to the circulating level of VLDL at lower rates of dietary cholesterol ingestion.

Following their removal by cells, the lipoprotein constituents are metabolized separately. The work of Quarfordt and Goodman (34) and the electron microscopic studies of Stein et al. (35), both using trace amounts of cholesteryl ester, suggested that this process requires several hours for completion. The present studies carried out over a wide range of remnant removal by the whole liver reveal the rate of hydrolysis of the lipoprotein cholesteryl ester to be slow relative to its rate of removal. It was also noted that saturation of hydrolysis was not approached in the range studied in whole liver and the plot of removed cholesteryl ester against hydrolyzed cholesteryl ester was not hyperbolic. These kinetics suggest that the rate of hydrolysis is not determined simply by the activity of the hydrolytic enzyme. One possibility is that the initial rate of hydrolysis is determined by the rate of diffusion of the remnant to the site where it is hydrolyzed rather than by the amount of hydrolytic enzyme in the liver.

This hypothesis requires that once the site of hydrolysis is reached, enough esterase is present to rapidly hydrolyze the cholesteryl ester. To explore this, the activity of the lysosomal cholesterol esterase which appears to be primarily responsible for cholesteryl ester hydrolysis (25, 26) was assayed. The activity of the lysosomal cholesteryl esterase was found to vary with the substrate used. Indeed the kinetics observed when dispersed cholesterol oleate was used as an enzyme substrate were different than when chylomicron remnants were used as a substrate. The enzyme had much higher affinity for the remnants but achieved a lower $V_{max}$ with them than with the artificial substrate. Comparison of the rate of hydrolysis in vitro and in whole liver was of further interest. If one assumes that in the whole liver all of the removed cholesteryl ester is available to the enzyme, then the rate of hydrolysis in vitro with either substrate is higher than the rate in whole liver. This becomes clear if the dotted lines in Figs. 4 and 5B are compared. In each case the concentration of cholesteryl ester that gave a hydrolysis rate of 0.5 $\mu$g per min per gm liver was estimated from the curve. In whole liver 165 $\mu$M cholesteryl ester from newly removed remnants was required to achieve this rate while in vitro only 4 $\mu$M cholesteryl ester in remnants was needed to achieve the same rate of hydrolysis. Although it is obviously difficult to extrapolate from in vitro enzyme measurements to the intact organ, one simple explanation for these observations is that in the intact cell only a small portion of the newly removed remnant is available to the enzyme, at least in the initial period after removal. This consideration, along with the finding that the rate of hydrolysis in the intact liver indicated kinetics compatible with a pas-
sive process, suggests that the initial rate of hydrolysis of cholesteryl ester from chylomicron remnants may be determined by the rate of diffusion of the particle to the site of hydrolysis. Since microtubules may have an important role in intracellular movement (36), this hypothesis is in accord with the report of Floren and Nilsson (26) that microtubule inhibitors block hydrolysis of chylomicron cholesteryl esters.

From these results it also becomes apparent that, following cholesterol ingestion, a hepatic pool of cholesteryl ester, which has not yet been released from the remnant, is created. The amount and rate of release of cholesteryl from this pool must be considered when attempting to interpret experiments concerning effects of cholesterol feeding on a hepatic metabolic process, such as the rate of hepatic cholesterol synthesis.16

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