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Studies on the Heterogeneity of Human Serum Lp Lipoproteins and on the Occurrence of Double Lp Lipoprotein Variants

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Lp lipoproteins have been prepared by a mild method from the serum of a large number of individuals. Approximately 25% of the individuals tested showed the presence of a double Lp peak in analytical ultracentrifuge diagrams. These double peaks were designated Lp(a)-1 and Lp(a)-2 to distinguish them from the single Lp(a) peak. The mean viscosity-corrected sedimentation coefficient, $s_{1.004,\ 20\ C}$, *and density of the single Lp(a) peak were* 15.8 ± 1.8s (n = *32) and* 1.076 ± 0.01 *g/ml, of the Lp(a)-1 peak were* 13.5 ± 1.1s (n = *14) and* 1.064 ± 0.007 *g/ml, and of the Lp(a)-2 peak were* 16.8 ± 1.7s (n = *14) and* 1.074 ± 0.009 *g/ml. Absorption tests using a double and single Lp preparation showed that both Lp peaks in the double variants possess Lp(a) specificity. Evidence is lacking as yet for individual specificities for either Lp(a)-1 or Lp(a)-2. Inter- and intra-individual heterogeneity among Lp lipoproteins is discussed.*

INTRODUCTION

The Lp(a)[3] lipoprotein was first described by Berg (1963). Genetic studies reviewed by Berg (1968) indicated that the presence or absence of the Lp(a)

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[3] The designation Lp lipoprotein refers to the human serum lipoprotein which carries the Lp(a) antigenic determinant. Individuals whose serum contains the Lp(a) antigen have the phenotypic designation Lp(a+). Individuals seemingly lacking Lp(a) are designated Lp(a−).

factor was under the control of a single autosomal gene. In 1970, Harvie and Schultz showed that all sera tested contained the Lp lipoprotein and suggested the quantitative nature of the genetic control. Other investigators have confirmed this finding (Utermann and Wiegandt, 1970). The Lp lipoprotein has been isolated from human serum by various methods involving precipitation with dextran sulfate, preparative and density gradient ultracentrifugation, and column chromatography (Wiegandt *et al.*, 1968; Schultz *et al.*, 1968; Ehnholm *et al.*, 1971; Simons *et al.*, 1970; Utermann and Wiegandt, 1969; Utermann *et al.*, 1972).

The Lp lipoprotein has a density between 1.064 and 1.12 g/ml, an $S_{f\,1.21,\,20\,c}$ of approximately 24, and an estimated molecular weight of 5×10^5 (Schultz *et al.*, 1968; Simons *et al.*, 1970; Ehnholm *et al.*, 1971). Preliminary biochemical data indicate that Lp lipoprotein has approximately 40% protein and a higher carbohydrate content than LDL[4] (Ehnholm *et al.*, 1972). The Lp lipoprotein shares some antigenic determinants with LDL, contains other(s) specific for Lp(a), and shows no cross-reactivity with HDL[4] (Schultz *et al.*, 1968; Wiegandt *et al.*, 1968). Recently it has been reported by two laboratories (Utermann *et al.*, 1972; Ehnholm *et al.*, 1972) that Lp lipoprotein disaggregated after standing, or after treatment with detergent, into three components, i.e., LDL lipoprotein, Lp(a) apoprotein, and albumin. The significance of this finding remains to be established.

During investigations in this laboratory of the preparation and analytical ultracentrifugal characteristics of the Lp lipoprotein, it was found that there was considerable interindividual heterogeneity among Lp lipoproteins isolated from a large number of people. It also became evident that Lp preparations from approximately 25% of a group of about 60 individuals showed a double peak in ultracentrifuge diagrams in the area ordinarily occupied by the single Lp peak. The occurrence of the double peak was found to be reproducible in samples of serum drawn and tested from a given donor at different times.

This report presents data to document the heterogeneity of the Lp lipoproteins from different individuals and to show that both the lipoproteins in the double variants carry Lp(a) specificity.

MATERIALS AND METHODS

Preparation of Lipoproteins

Sera used in this study were purchased from institutionalized, healthy, male donors, of both the Caucasian and Black races. The men ranged in age from 24 to 53 years, they were all on the same diet, they were not hyperlipemic, and they were not undergoing any drug treatment.

[4] LDL refers to low-density or β-lipoprotein. HDL refers to high-density or α-lipoprotein.

Lp lipoprotein samples were prepared according to the method of Harvie and Schultz (1970). This method is considered to be mild as it minimizes manipulation, consisting of one dextran sulfate precipitation step, two dialyses, and one gradient fractionation in the preparative ultracentrifuge. Samples were taken from the density gradient and dialyzed against the appropriate NaBr solution for analytical ultracentrifugation.

Analytical Ultracentrifugation

Analytical ultracentrifugation was done in a Beckman Model E ultracentrifuge using the An-D rotor at 20 C either at 30,000 rpm ($65{,}403 \times \bar{g}$) for runs at 1.21 g/ml density or at 60,000 rpm ($261{,}612 \times \bar{g}$) for runs at 1.004 g/ml density. All sedimentation coefficients (s) are in Svedberg units and were corrected for solvent viscosity, but not for the density.

Density and Viscosity

The density of the salt solutions used for gradients and analytical ultracentrifugation was determined by measuring the dry weight of the solutions and combining these data with density data in the *International Critical Tables* (1928). Viscosity measurements were made according to the method of Ostwald (Findlay, 1935).

Antisera

Specific Lp(a) antisera were prepared, absorbed, and checked by gel diffusion analysis as described previously (Schultz *et al.*, 1968). Two antisera were used, one prepared against a double Lp variant, called anti–double Lp, and another prepared against a single Lp variant, called anti–single Lp.

Concentration of Antisera

Even though the titer of both antisera against the Lp antigen was high, it was not sufficiently high to absorb all the Lp activity of the Lp preparations used for the absorption tests. Therefore, both antisera were concentrated by partially purifying the globulins by precipitation for 1 hr at room temperature with saturated ammonium sulfate. The precipitate was redissolved in 0.15 M NaCl in a smaller volume than the original sample and then dialyzed against 0.15 M NaCl.

Absorption Tests

Lp lipoprotein samples were concentrated twofold by pervaporation for the

absorption tests so that after dilution in the test proper the final concentration of the Lp lipoprotein, if present, would be equal to that of the original isolated preparation. The antigen was dialyzed against 0.15 M NaCl. Absorption was done by mixing equal volumes of Lp preparation and antiserum and then incubating the mixture for 1 hr at 37 C followed by 24 hr at 4 C. The precipitate was removed by centrifugation at 2000 rpm in a clinical model table centrifuge for 30 min.

The absorbed supernatant was dialyzed against 1.12 g/ml density NaBr overnight. Gradients were made for Lp isolation as was described under "Preparation of Lipoproteins" (Harvie and Schultz, 1970) except that the amount of solution of each layer was exactly scaled down from the 13 ml total volume of the No. 40 Spinco rotor tube to the 5 ml volume of the SW39 Spinco rotor tube. The gradients were centrifuged 24 hr at 35,000 rpm (100,000 $\times \bar{g}$) in the Spinco Model L ultracentrifuge. Tubes were cut to isolate the Lp lipoproteins at a position comparable to the Cut 6–9 of the No. 40 tube gradients which contained the Lp lipoprotein. Samples were dialyzed against 1.004 g/ml density NaBr and subjected to analytical ultracentrifugation at 60,000 rpm, 20 C.

Statistical Procedures

Correlation coefficient (r) was calculated according to Hald (1952, p. 89), and variance and standard deviation (SD) according to Hald (1952, p. 72). Intra-individualized variance and SD were calculated for sedimentation coefficient data consisting of multiple Lp samples from a group of individuals each with a different mean but each individual tested in replicate (Hald, 1952, p. 426).

RESULTS

Evidence for Double Variants of the Lp Lipoprotein

During the investigation of over 100 Lp lipoprotein preparations, we have found that certain individuals showed the presence in the ultracentrifuge diagrams of two peaks sedimenting faster at density 1.004 g/ml than the accompanying β-lipoprotein. We had visual evidence from our earlier studies that the Lp band in the preparative gradients might be double, but it was not until we started our present method of isolation using a minimum number of steps that the evidence was found in analytical ultracentrifuge diagrams. So far, of 34 different individuals whose sera were specifically examined for the presence of the two Lp lipoproteins, nine (26.5%) have shown the presence of the double peak. Figures 1 and 2 show ultracentrifuge diagrams and viscosity-corrected sedimentation coefficients ($s_{1.004,\ 20\ C}$) for these nine individuals. The Lp peaks are the two right-hand peaks in each diagram. The left-hand

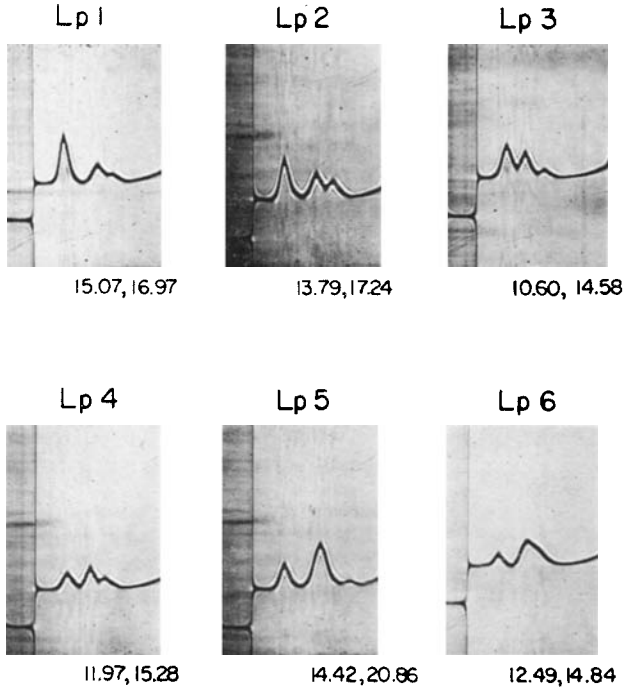

Fig. 1. Analytical ultracentrifuge diagrams (t = 32 min) of six double Lp preparations. Viscosity-corrected sedimentation coefficients at 1.004 g/ml density, 20 C, 60,000 rpm, are given below each Lp peak but not below the slow accompanying β-lipoprotein peak.

peak is the accompanying β-lipoprotein in the preparation, the amount of which varies from preparation to preparation. As indicated (Fig. 2), Lp 7-1, 7-2, 7-3, and 7-4; Lp 8-1 and 8-2; and Lp 9-1 and 9-2 are replicate Lp isolations from three individuals made at least a month, and often more, apart. It is notable that among individuals the two Lp peaks in the ultracentrifuge diagrams of the Lp preparations show variation both in sedimentation rate and in amount relative to each other. These variations do not exist, however, or are at a minimum, in the sera from a single individual drawn and tested over a period of time. In this paper, the slower Lp peak (middle peak in the diagrams) will be referred to as Lp(a)-1 and the faster peak as Lp(a)-2.

After discovery of the double Lp lipoproteins, it was of interest to determine if both peaks in the Lp region of the ultracentrifuge diagrams did have Lp(a) specificity and if antisera made against a double Lp variant contained antibodies specific to either of the two Lp lipoproteins.

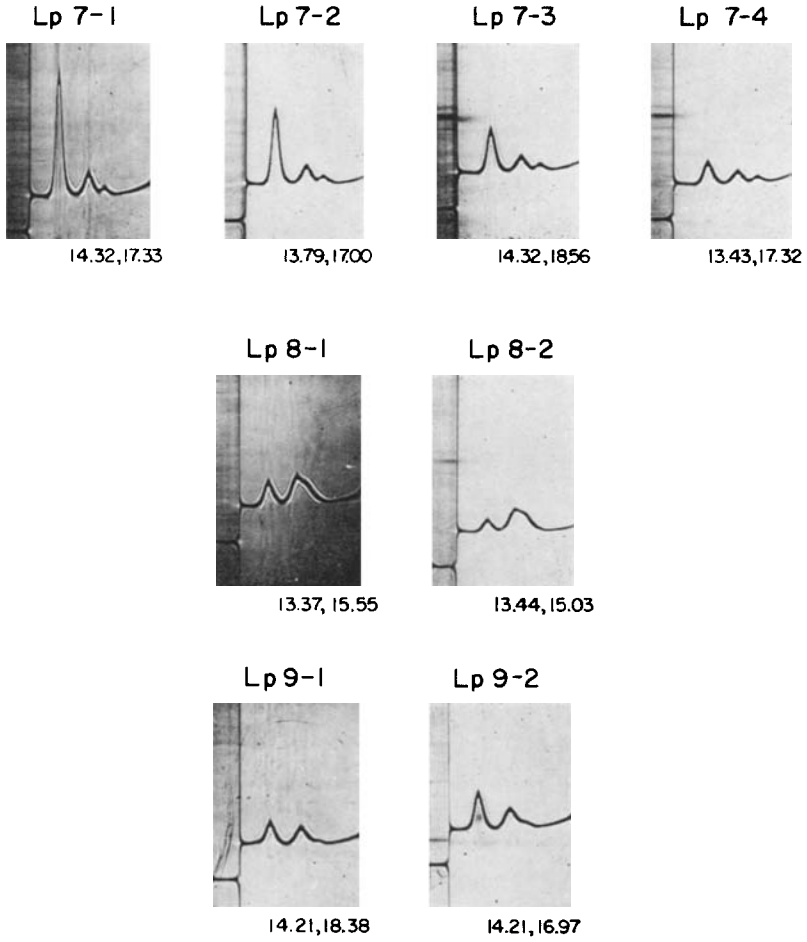

Fig. 2. Analytical ultracentrifuge diagrams (t = 32 min) of eight double Lp preparations from three individuals, 7, 8, and 9, tested in replicate over extended periods of time. Viscosity-corrected sedimentation coefficients at 1.004 g/ml density, 20 C, 60,000 rpm, are given below each Lp peak but not below the slow accompanying β-lipoprotein peak.

The occurrence of the Lp(a) determinant on both Lp lipoproteins was investigated experimentally using the following absorption tests. Antiserum was prepared against the double Lp preparation shown in Fig. 3A (3B is a diluted sample of 3A) and was called anti–double Lp. Antiserum was also prepared against the single Lp preparation shown in Fig. 3C and was called anti–single Lp. The slow peak in the diagrams represents the accompanying

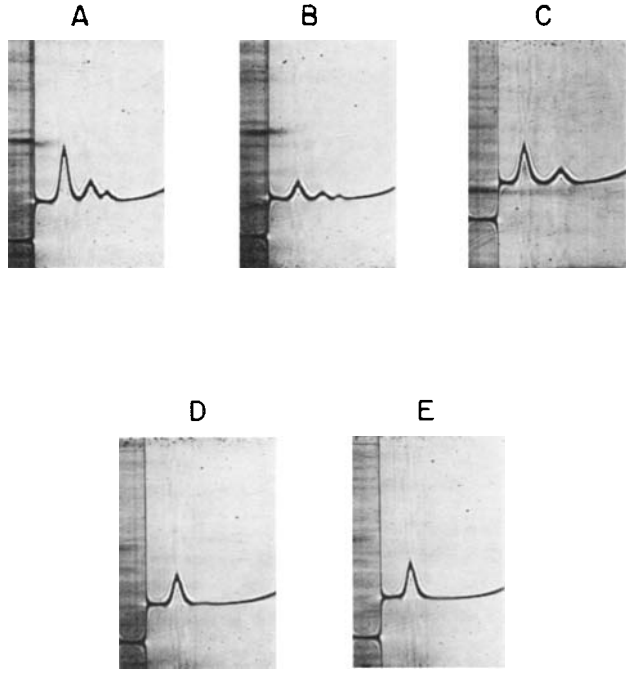

Fig. 3. Analytical ultracentrifuge diagrams, 1.004 g/ml density, 20 C, 60,000 rpm, t = 28 min, of (A) double Lp preparation A, (B) double Lp preparation A diluted 1:3, (C) single Lp preparation C, (D) double Lp preparation A absorbed with homologous antiserum, and (E) double Lp preparation A absorbed with antiserum against single Lp preparation C.

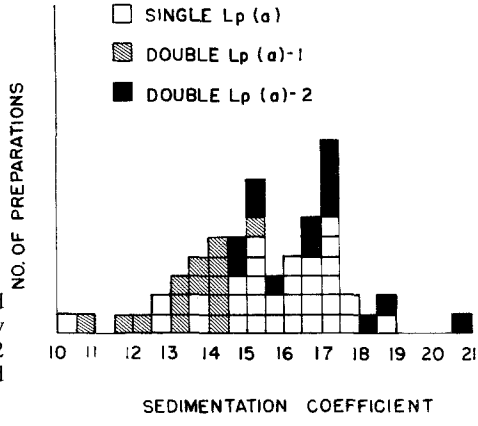

Fig. 4. Distribution of viscosity-corrected sedimentation coefficients at density 1.004 g/ml, 20 C, 60,000 rpm, of 32 single Lp lipoprotein preparations and 14 double Lp lipoprotein preparations.

β-lipoprotein. Antisera made against these preparations were absorbed with β-lipoprotein prepared from an Lp(a)-"negative," i.e., Lp(a−), individual so they had only antibodies of the Lp specificity present. A double Lp preparation (Fig. 3A) was then absorbed with anti-double Lp and another sample of it with anti-single Lp. Figure 3D shows the double Lp preparation absorbed with anti-double Lp, and Fig. 3E shows the double Lp preparation absorbed with anti-single Lp. The removal of both Lp lipoproteins in the double Lp preparation by anti-single Lp serum indicates that Lp(a)-1 and Lp(a)-2 share a common Lp determinant, provided that the single Lp preparation was indeed single and did not contain any of the second Lp molecular species, which had not been detected by our methods.

The presence of an antigenic determinant on one of the double Lp lipoproteins not shared by the other was investigated using an antiserum prepared against a double Lp preparation. Samples of this antiserum were absorbed with a number of different single Lp preparations. If the anti-double Lp antiserum contained antibodies of two different specificities, only one of which was directed toward the Lp lipoprotein in the single Lp preparation used for absorption, then the antibodies of the second specificity should remain in the antiserum after the absorption procedure and be free to react with one of the Lp lipoproteins present in a double Lp preparation which is used to detect it. In none of a number of such experiments was there any evidence of any residual antibody in the anti-double Lp antiserum after absorption with single Lp preparations. There were several factors which may have affected the results of this experiment. When our anti-double Lp serum was made, we were not aware that we were immunizing with a double variant, and the manner of preparation of the double Lp antigen might have reduced the concentration or eliminated the second Lp. Therefore, the resulting antiserum might not have had antibodies with the new specificity. Furthermore, if the second Lp is a quantitative trait like Lp(a) (Harvie and Schultz, 1970), the increased concentrations of absorbing antigen needed to assure removal of one molecular species of Lp might have had a sufficient amount of the material from the second species to afford absorption even by what seemed to be a single Lp variant. There is a possibility that any given single Lp may be either Lp(a)-1 or Lp(a)-2, and therefore absorptions with different single Lp preparations should be done.

The Heterogeneity of Lp Lipoproteins

The viscosity-corrected sedimentation coefficient ($s_{1.004,\ 20\,c}$) of 60 Lp lipoproteins, including 14 double variants, had a distribution shown in the bar graph of Fig. 4. The mean $s_{1.004,\ 20\,c}$ of the Lp peak from the single variants was 15.8 ± 1.8 ($n = 32$), of the Lp(a)-1 peaks was 13.5 ± 1.1, and of the

Lp(a)-2 peaks was 16.8±1.7. Although the distribution is quite broad, this breadth is probably not due to artifacts of preparation as the SD of 26 replicate determinations on Lp preparations made from ten individuals at times separated by a month or more was only 0.5*s*.

The distribution in Fig. 4 is bimodal, accentuated by the presence of the double variants. There is almost no overlap of Lp(a)-1 and Lp(a)-2, and it is interesting to speculate that in individuals having only one Lp peak this peak may turn out to be of either the Lp(a)-1 or Lp(a)-2 variety.

An attempt was made to study the density distribution of Lp preparations. The method used was to determine the sedimentation coefficient at 1.004 g/ml density and the flotation coefficient at 1.21 g/ml density. Densities of the Lp lipoproteins were calculated by extrapolating the data to the point where the sedimentation coefficient was zero. The data obtained are only approximations due to the fact that our Lp preparations always contain accompanying β-lipoprotein. Although the separation of this β-lipoprotein from Lp in the analytical ultracentrifuge was clear-cut at density 1.004 g/ml, at density 1.21 g/ml the density differences between the β and the Lp were "washed out" and the two floated as one peak in most cases. The mean density for 19 preparations of single Lp was 1.076±0.01 g/ml, with a range of 1.060–1.094 g/ml. The mean densities for eight preparations of Lp(a)-1 and Lp(a)-2 were 1.064±0.007 g/ml and 1.074±0.009 g/ml, respectively. Previous experiments on two preparations made by multiple gradient fractionation which contained only one Lp lipoprotein and no accompanying β-lipoprotein and in which densities were determined by sedimentation at two densities and flotation at two densities gave values of 1.068 g/ml and 1.070 g/ml. That the figures presented here are only approximations is indicated by the fact that the average density of eight Lp(a)-2 lipoproteins was 1.074 g/ml, which seems low considering the mean sedimentation coefficient of Lp(a)-2, which is 16.8*s*.

Theoretically, there should be a near perfect correlation between sedimentation coefficient and density provided one is not dealing with an associating system. The correlation coefficient (r) calculated between 33 $s_{1.004,\ 20\,C}$ values and densities was 0.68, again pointing either to the approximate nature of the data or to the operation of unknown factors. If the correlation coefficient is calculated using data from single Lp variants, it is only slightly higher, i.e., 0.71.

DISCUSSION

It has been apparent to investigators of Lp lipoprotein that this factor is very labile, denaturing readily during experimental manipulation (Harvie and Schultz, 1970; Simons *et al.*, 1970). For this reason, we have chosen a method of preparation of Lp lipoprotein which minimizes manipulation, consisting of one precipitation step with dextran sulfate, two dialyses, and one gradient

fractionation in the preparative ultracentrifuge. Preliminary studies showed that the ultracentrifuge diagrams of Lp lipoprotein isolated by dextran sulfate precipitation did not differ from diagrams of Lp lipoprotein isolated by multiple preparative ultracentrifugal steps. Results of isolation of over 100 Lp preparations using these methods showed that the so-called accompanying β-lipoprotein is not eliminated. Our studies have been done on samples containing this impurity, as it was evident that the Lp lipoprotein was readily distinguishable from it. We acknowledge that the presence of accompanying β-lipoprotein probably influences the precise sedimentation rates and especially the flotation rate at density 1.21 g/ml.

The different rates of sedimentation of the two Lp lipoproteins in the analytical ultracentrifuge could be due to the presence of two molecular species of Lp with identical composition of lipid and protein moieties but different molecular weights due to an association phenomenon. On the other hand, there could be present two molecular species of differing percentage of lipid and protein which would thus have different densities (and different molecular weights) and different sedimentation rates. Our preliminary density studies favor the latter explanation. At present, the knowledge of the chemical composition of Lp lipoproteins is more qualitative than quantitative. More precise data will be available only when Lp lipoproteins are obtained purified in larger amounts than are available now in a physical and chemical state approximating that which occurs *in vivo*.

Reports have appeared indicating that seemingly purified Lp lipoprotein can be obtained by additional column chromatography (Simons *et al.*, 1970; Ehnholm *et al.*, 1971; Utermann *et al.*, 1972) and/or polyacrylamide gel electrophoresis (Garoff *et al.*, 1970; Utermann and Wiegandt, 1970). No data have been presented which characterize these preparations by analytical ultracentrifugation or give chemical characteristics of multiple samples. Due to the cross-reactivity of Lp and β-lipoprotein, antigen–antibody reactions are not sufficient proof of homogeneity.

That our preparations are adequate for a study of characteristics of multiple preparations from the same and different individuals is substantiated by an intraindividual SD of the $s_{1.004,\ 20\,C}$ of 0.5*s*, which is one-fourth to one-third of the SD of *s* of interindividual preparations.

The appearance of the double Lp lipoprotein variants poses the possibility that we have discovered another lipoprotein polymorphism in human serum. So far, it is one without a specific antibody different from Lp(a), and therefore more effort is indicated to elucidate its specificity. The Lp(a)-2 reported here could be a multimer of Lp(a)-1. It is not likely to be one of the disaggregation products reported by Utermann *et al.* (1972), as there is no evidence for non-lipid-containing components. The consistent finding of the double peaks in certain individuals argues for their natural occurrence.

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