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Structural Changes in Plasma Circulating Fibrinogen after Moderate Beer Consumption as Determined by Electrophoresis and Spectroscopy

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The effects of short-term moderate beer consumption (MBC) on plasma circulating fibrinogen (PCF) in patients suffering from coronary atherosclerosis were investigated by use of 2-dimensional electrophoresis (2-DE), circular dichroism (CD), and Fourier transform infrared spectroscopy (FT-IR). Forty-eight volunteers after coronary bypass surgery were divided into experimental (EG) and control (CG) groups, each of 24. Patients of the EG group consumed 330 mL of beer/day (about 20 g of alcohol) for 30 consecutive days, and CG volunteers drank mineral water instead of beer. Blood samples were collected before and after the experiment. In 21 out of 24 patients after beer consumption the plasma circulating fibrinogen was compromised: changes in its secondary structure were found. These changes were expressed in relatively low electrophoretic mobility and charge heterogeneity, decrease in α-helix and increase in β-sheet, and in slight shift of amide I and II bands. Our findings indicate that one of the positive benefits of moderate beer consumption is to diminish the production of fibrinogen and its stability, which reduces the potential risk exerted by this protein. Thus, in most of beer-consuming patients some qualitative structural changes in plasma circulating fibrinogen were detected.

KEYWORDS: Moderate beer consumption; fibrinogen; CD; FT-IR; 2-DE; structural changes

INTRODUCTION

Alcoholic beverages are an integral part of diets in the West. At present, alcoholic beverages account for about 4−6% of the average energy intake (*1*). It was demonstrated that moderate alcohol consumption is cardioprotective: it positively influences plasma lipid levels and antioxidant and anticoagulant activities in patients suffering from coronary artery disease (CAD) (*2*−*4*). This was attributed to the chemical and biochemical composition of alcoholic beverages, including beer. Total polyphenols and flavonoids (epicatechin and quercetin) are the main components of beer antioxidants, which influence the lipid and protein metabolism. The amount of these components depends on the technology and raw materials used during beer preparation (*3*−*6*). However, alcohol consumption has some adverse effects: inter alia, it negatively influences protein metabolism (*7*, *8*), including plasma circulating proteins (*9*−*11*). Fibrinogen is one of the plasma proteins. Evidence links fibrinogen with coronary atherosclerosis and blood coagulation (*2*, *12*). Mennen et al. (*12*) claim that moderate drinking may lead to a decrease in plasma circulating fibrinogen concentration (PCF). Could moderate alcohol consumption lead also to structural changes in PCF? As far as we know, there are no

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investigations that have connected alcohol consumption and qualitative changes in PCF. Therefore, we decided to conduct prospective investigations involving fibrinogen structural studies and moderate beer consumption. In our previous investigations, intrinsic fluorescence (IF), differential scanning calorimetry (DSC), and hydrophobicity tests were applied to study different plasma fractions (*1*, *10*, *11*). This report describes the results of the structural changes in PCF determined by two-dimensional electrophoresis (2-DE), circular dichroism (CD), and Fourier transform infrared (FT-IR) spectroscopy.

MATERIALS AND METHODS

Materials. Maccabee beer samples were kindly supplied by Tempo Beer Industries, Natania, Israel. Their major components were proteins (5.2 g/L), total sugars (20.5% on dry substance), alcohol (5.1% volume), total polyphenols (345 mg/L), epicatechin (65.5 mg/L), and quercetin (0.95 mg/L) (*5*, *6*).

Fibrinogen was precipitated by methanol and then purified by sequential DEAE anion-exchange chromatography, dialyzed against water for 72 h, and lyophilized. Proteins were dissolved in 0.01 M phosphate buffer, pH 7.2. Human plasma fibrinogen (F4883) was purchased from Sigma Chemical Co., St. Louis, MO, as were all other chemicals.

Subjects. The study population was recruited from patients/volunteers who have previously undergone coronary bypass surgery due to CAD in the Institute of Cardiology of the University Medical Center, Rehovot, Israel. The subjects gave written, informed consent to a protocol approved by the responsible Institutional Committee on human experimentations based on the Helsinki Declaration of 1975 as revised in 1983.

Forty-eight male patients between ages of 46 and 72 years were examined. They were randomly divided into beer-consuming experimental (EG) and beer not consuming control (CG) groups, 24 each. All have consumed a Mediterranean-type diet rich in vegetables and fruits with limited quantities of fats as recommended for CAD patients. For 30 consecutive days this diet was supplemented once a day by 330 mL of Maccabee beer (about 20 g of alcohol) for EG group patients. CG group during this 30 days period instead of beer drank 330 mL of mineral water Netivot.

Methods. As was stated in the Introduction section, to detect possible qualitative changes in the plasma circulating fibrinogen after beer consumption, the following procedures were used.

Two-Dimensional Electrophoresis. 2-DE was performed following the procedure of Otto et al. (*13*). The protein sample was dissolved in lysine buffer containing 9 M urea, 70 mM DTT, and 2% ampholyte mixture (Servalyte 2-4), 25 mM Tris-HCl, pH 7.1, 50 mM KCl, 3 mM EDTA, 2.9 mM benzamidin, and 2.1 μM leupeptin. Then the sample at a concentration of 33 μg/μL was applied to isoelectrofocusing (IEF) in the first dimension. The IEF gels contained 2% of an ampholyte mixture at pH 2−11. In the second dimension SDS−PAGE was used with 15% polyacrylamide gels according to Laemmli (*14*). Molecular mass standards of trypsin inhibitor, soybean (20 kDa), myoglobin (30 kDa), alcohol dehydrogenase (50 kDa), and 70 kDa were obtained from Sigma Chemical Co. The 2DE-gel size was about 7−8 cm. Gels were stained with 0.25% Coomassie Brilliant Blue R 250 in methanol/water/acetic acid and destained in the same solvent. The scan of the gels was done with a laser densitometer (Bio-Rad, Fluor-S, Multi-Imager) and images were analyzed with the software (Bio-Rad Multi-Analyst TM/PC, version 1.1). For each spot, adjusted volume [optical density (OD) $\times$ area of the spot (in square millimeters)] was used as a measure of relative concentration. Statistical analyses were based on Student's *t*-test, which was included in the software.

Circular Dichroism. CD spectra were measured on a Jasco J-600c spectropolarimeter (Japan Spectroscopic Co., Ltd.), with a 0.05 cm quartz cell at room temperature under constant nitrogen purge. Solutions (0.01 mg/mL) of fibrinogen were prepared by dissolving the lyophilized sample in 0.01 M phosphate buffer, pH 7.2. The absorbencies of all solutions were kept below 1.0 (*11*, *15*). CD spectra represent an average of eight scans collected in 0.2 nm steps at a rate of 20 nm/min over the wavelength range 180−250 nm of far-UV (FUV). To compare the stability of proteins before and after beer consumption, the denaturation was done with 8 M urea. CD spectra were baseline-corrected, and the data are presented as the mean residue ellipticities (θ). The CD spectra were evaluated with Contin software according to Provencher's algorithm, allowing the comparison of secondary structures from different proteins (*16*).

Fourier Transform Infrared Spectroscopy. A Bruker Optic GMBH Vector FT-IR spectrometer (Bruker Optic GMBH, Attingen, Germany) was used to record IR spectra. Lyophilized material was mixed with KBr, and the pellet was pressed at 10 000 kg/cm^2 for 15 s. A potassium bromide microdisk was prepared from finely ground powder of 2 mg of lyophilized sample or standard fibrinogen with 100 mg of KBr.

Statistical Analysis. To verify the statistical significance of studied parameters, means (M) of three times analyzed samples $\pm$ SD were defined. Differences between groups were established by use of 2-way ANOVA. The p values of <0.05 were considered significant.

RESULTS AND DISCUSSION

After 30 days of beer consumption, only in patients of the EG group was an improvement in lipid metabolism and antioxidant and anticoagulant activities registered (*2*). There were not significant changes in the concentration of plasma proteins, including PCF. However, after 30 days of beer consumption, in 21 out of 24 patients of the EG group qualitative changes in plasma circulating fibrinogen were discovered. These typical changes in plasma fibrinogen of the patient N are described below.

Electrophoresis. Experimental plasma circulating fibrinogen (PCF) before and after moderate beer consumption (MBC) was separated by 2-DE (**Figure 1**, panels **A** and **B**, respectively), and the charge heterogeneity was observed. The obtained 2-DE patterns were compared with the computer-generated one by use of Swiss-2D PAGE Map Selection of human fibrinogen proteins, GIF image, and expasy right-plasma website: www.expasy.ch/ch2dgifs/publi/plasma-basic.g. Fibrinogen has migrated mostly in several bands with apparent molecular masses of about 10, 20, 30, 50, and 70 kDa. The proteins in the range from 100 to 200 kDa were not investigated in this report.

In all gels the majority of proteins migrated with a molecular mass of ca. 35−70 kDa, differing in their isoelectric points over a broad range of p*I* 4.0−10.0. Fibrinogen had aggregated subunits mostly in high molecular weight around 50−70 kDa. In addition, some small polypeptides in the basic region (p*I* ca. 8−10) and a few low molecular weight proteins in the acidic region (p*I* ca. 3−5) were visible in the plasma of patients. The main band of ca. 50 kDa resolved here into a pearl-chain-like series of proteins with p*I*s of ca. 5−10, whereas the lower protein band consisted of some pronounced strongly acidic protein spots of ca. p*I* 6.5 and additional components spreading over the entire pH range.

Two spots at U_1 position (p*I* 4.5−5.5 and MW 10 kDa), two spots at U_2 position (pI 6.5−7.0 and MW about 30 kDa), and one spot at U_3 position (p*I* 4.8 and MW 48 kDa) which were found on the gel before beer consumption (**Figure 1A**) have disappeared from the fibrinogen plasma sample after beer consumption (**Figure 1B**). Under denaturing conditions, when unfolding takes place, the proteins differ in shape and size. Folded proteins are remarkably compact in comparison to a random polypeptide. The shape of a protein affects the rate at which it migrates through polyacrylamide gels. The comparison with the data spots online (**Figure 1C**) has shown similar distribution of the proteins, but the amount of the applied sample

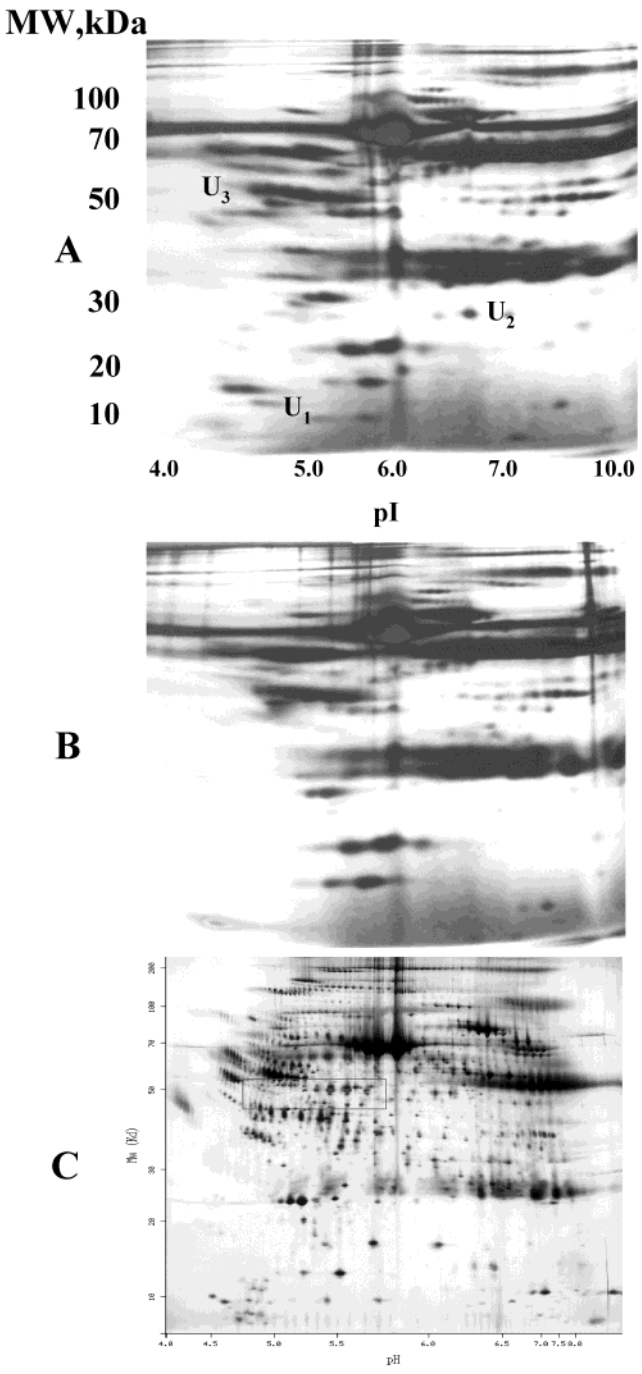

Figure 1. Two-dimensional electrophoresis (2-DE) of plasma circulating fibrinogen (PCF) from patient N before (**A**) and after (**B**) moderate beer consumption (MBC). (**C**) PCF of healthy normal patient online: at the SWISS-2DPAGE site (http/www.expasy.ch/cgi-bin/ch2d-compute-map?PLASMA_HUMAN, PO2679). The vertical axis shows the molecular masses of proteins in kilodaltons, and the horizontal axis shows the distribution of proteins by their isoelectric points. U, unknown proteins.

protein was lower in the online 2-DE than in our experiment. These results are in accordance with others that fibrinogen is composed of three pairs of nonidentical chains (α, β_2, and γ_2) with molecular masses of 67, 57, and 47 kDa, respectively, interconnected by a complicated series of disulfide bonds (*17*, *18*).

Our results are in agreement with others (*19*−*21*), who showed two fragments of 45 000 and 100 000 in fibrinogen after treatment with plasmin, and in the fragment of 100 000 was also a globular protein of 27 000.

Scanning of SDS protein patterns and the calculation of the percentage of volume of each spot demonstrates difference in intensity of the spots in the low molecular weigh before and after beer consumption (**Figure 1A,B**). The adjusted volumes of common spots at 30 kDa (pI = 5.3) before moderate beer consumption were 0.41 and 0.49, in comparison with the corresponding ones after moderate beer consumption of 0.32 and 0.27. At 25 kDa and pI 5.5−6.2, three spots showed adjusted volumes of 0.72, 0.84, and 0.11, in comparison with the corresponding ones after moderate beer consumption of 0.67, 0.75, and 0.14. It was a slight decrease in the adjusted volumes after beer consumption in the corresponding common spots.

After beer consumption, new interchain disulfide bonds may be formed. As a result, aggregated forms were received in the spots, corresponding to molecular mass 38−40 kDa. Smaller quantity of spots but broader ones with larger molecular weight of proteins are shown after beer consumption (**Figure 1B**).

Fibrinogen circulating in human blood is composed of high molecular weight (HMW) and lower molecular weight (LMW) fractions (*22*). Before the investigation the electrophoretic images of plasma circulating fibrinogen in both groups of patients were identical without any detectable differences. After the 30-day period of moderate beer consumption, in 21 out of 24 patients of the experimental group some qualitative changes in separated plasma proteins were found.

According to our results, changes after beer consumption have appeared only in LMW proteins. Degradation of a polypeptide could theoretically shift the isoelectric focusing point rather dramatically, but simulated degradation of either the N- or C-terminal ends of the γ-chain (up to 12 residues) and succeeding calculation of pI with EXPASY Compute pI and MW did not result in the extreme changes required to explain the group of rather basic γ-chains. We have therefore concluded that measurement of this fraction of fibrinogen may prove to be of clinical diagnostic significance. The present study is an attempt to characterize and explain the heterogeneity of human fibrinogen (*22*−*26*). Electrophoretic separation of human serum proteins does not give the estimation for their classification or stability, but it is an additional index for their partial characterization.

Circular Dichroism. The far-UV CD spectra for different states of fibrinogen (native and denatured with ethanol under the influence of beer consumption) are shown in **Figure 2**. The CD of native fibrinogen spectrum (**Figure 2**, curve 1) shows a negative peak as well as the denatured one with ethanol (**Figure 2**, curve 2) centered around 222 nm. These bands demonstrate a significant α-helical content of fibrinogen. Another smaller band at 213 nm is characteristic for fibrinogen with significant quantities of β-structure. A negative maximum around 202 nm is typical for a protein with a disordered random coil structure. Urea causes the protein to undergo a disorganization of its secondary structure involving a complete helix-to-coil transition. Unfolding of fibrinogen is a result in promoted interactions between exposed functional groups, which involve transconformations of decrease in α-helix and increase in β-sheet when denatured with ethanol of beer-consuming patient (**Table 1**). Denaturation with 8 M urea brought an even higher increase in β-sheet content, at the expense of α-helix (**Table 1**). Urea denaturation of fibrinogen leads to a decrease in α-helical content to 15% ,compared to 40% in the native state. The

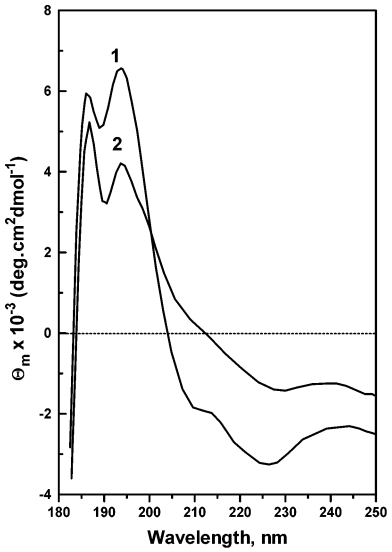

Figure 2. Circular dichroism (CD) spectra of plasma circulating fibrinogen (PCF). Curve 1, bands of PCF before moderate beer consumption (MBC); curve 2, bands of denatured PCF after MBC. Samples of concentration 0.01 mg/mL were dissolved in 0.01 M phosphate buffer. Each spectrum was run at room temperature.

Table 1. Structural Properties of Native and Denatured Plasma Circulating Fibrinogen[a]

proteins	α-helix	β-sheet	R
Before Moderate Beer Consumption			
PCF	40 ± 3.9	19 ± 2.1	41
PCF + 8 M urea	27 ± 2.5	29 ± 2.6	44
After Moderate Beer Consumption			
PCF	31 ± 3.2	24 ± 2.3	45
PCF + 8 M urea	15 ± 1.4	32 ± 3.2	53

[a] Values are means ± SD of triplicates; R, random coil.

spectrum of fibrinogen does not closely resemble that of the native state and demonstrates the much less pronounced negative maxima at 222 and 208 nm typical of α-helix. The far-UV spectrum of the ethanol-denatured fibrinogen shows a spectrum similar to that of the native protein. However, upon denaturation with ethanol the decrease in α-helical content in comparison to the native state was also observed.

CD studies suggested that the α-helix content decreased from 42% in its native state to 29% in the cis-DDP-treated form (*23*). Our results are in good agreement with others, who used circular dichroism spectroscopy (*19*). Fragment E with MW 45 000 is shown to contain 50% α-helical values, attributed to its coiled-coil portions, and minor β-strands and turn structures. On the other hand, fragment D, MW 100 000, contains a distribution of secondary structure values of 35% α-helix, 29% β-sheet segments, and 17% turn structures. Fragment D itself has two domains: a portion of the original coiled-coil and also a thermally labile globular domain to be rich in β-sheet structures (*19*). The coiled-coil portion (MW 27 000) showed a high α-helical content (around 70%). The extent of the fluorescence quenching and the decrease in helix correlated with the number of cleaved S−S bonds. The results indicate secondary conformational alteration of PCF involving a structural perturbation of nearby tryptophan residues through S−S bond cleavage (*23*), which is in accordance with our results under denaturation conditions (*1*, *10*, *11*). In general, these results clearly show that denaturation of plasma proteins precedes gel formation, which is in line with the two-stage mechanism (*27*). Similar

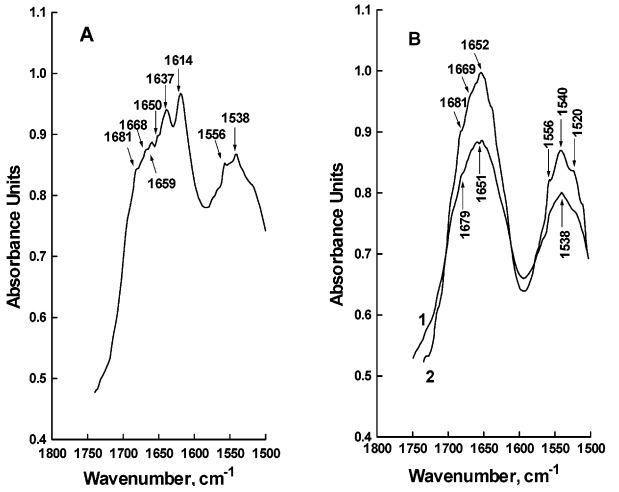

Figure 3. Absorption spectra from 1800 to 1500 cm^{-1}. (**A**) Plasma circulating fibrinogen (PCF) (F4883 standard). (**B**) Experimental PCF: curve 1, from patient N before moderate beer consumption (MBC); curve 2, from the same patient after MBC.

biphasic features were observed earlier and confirmed the existence of a stable intermediate conformation. According to the two-state model, which applies to small globular proteins after the rapid disappearance of ordered structures (mostly α-helical), there still remains a large part of folded protein molecule. These molecules unfolded during the subsequent denaturation (*27*, *28*). Circular dichroism analysis revealed a shift in the mean residual ellipticity and a reduction of α-helix content after beer consumption, which is in accordance with others (*20*).

FT-IR Spectroscopy. The secondary structure of human fibrinogen has been studied by FTIR spectroscopy. The qualitative results for fibrinogen are in good agreement with circular dichroic spectroscopy (*29*, *30*). CD studies of fibrinogen gave a value of 40% α-helices and the presence of minor segments of β-sheets (18−20%). Our results on CD measurements are in agreement with others (*19*, *30*, *31*), who calculated the composition of the secondary structure, detected an amide I/amide II ratio in fibrinogen spectra with some changes in the relative intensity of the bands from FT-IR data. All fibrinogen samples showed similar bands at amide I (AI), amide II (AII), and amide III (AIII) bands (in the range of 1650, 1530, and 1300−1250 cm^{-1}) but differ slightly for spectra after beer consumption. The spectrum of standard fibrinogen (**Figure 3A**) showed the amide I band at 1650 cm^{-1}. The sample of fibrinogen of the patient N before (**Figure 3B**, line 1) and after (**Figure 3B**, line 2) beer consumption showed broad amide I bands at 1652 and 1651 cm^{-1}, typical of proteins with high α-helical content.

The rather high ratio of AII to AI bands in the sample before beer consumption can be attributed to the high content of α-helix. Some other spectral bands were located at frequencies of 1681, 1669, 1659, 1637, and 1614 cm^{-1}. These numbers were found also in the standard spectrum of fibrinogen. The band at 1651−1652 cm^{-1} originates from α-helical and/or random structures. The band of high-frequency components in the amide I band at 1669 cm^{-1} can be assigned to turns and elements of β-sheet (*19*, *30*). Amino acid side chains such as those from Arg, Asn, Gln, and Lys absorb along the whole amide I band and may affect specially the intensity of the latter two bands and that at 1630 cm^{-1} (see a band with very low intensity on line 1, **Figure 3B**), and their whole contribution to the amide I

and II bands is estimated to be around 20% of the total absorbance. The amide II band is shown at 1556 and 1540 cm^{-1}. The differences in the FT-IR data between the sample before and after beer consumption were shown only in a small shift of the bands of amides I and II, characterizing the decrease in α-helical content.

Comparison of all serum fractions before and after MBC showed the same range of changes in serum albumin and globulin. Only in the fibrinogen fraction was registered a bigger fluorescence shift than in serum albumin and globulin (this fraction was more stable). The change in the intensity of all samples after beer consumption can be explained by the structural changes of proteins (*1*, *10*, *11*).

In conclusion, after short-term moderate beer consumption in 21 out of 24 CAD patients, qualitative changes in the plasma circulating fibrinogen were found: (a) differences in distribution of protein spots, (b) changes in functional properties such as the position of amide I and II bands, and (c) changes in the secondary structure composition: decrease in α-helix as an indicator of protein stability.

Fibrinogen plays an important role in the coagulation process, and therefore these finding have to be further explored.

ABBREVIATIONS USED

CD, circular dichroism; CG, control group; EG, experimental group; FT-IR, Fourier transform infrared spectroscopy; MBC, moderate beer consumption; PCF, plasma circulating fibrinogen; 2-DE, two-dimensional electrophoresis.

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