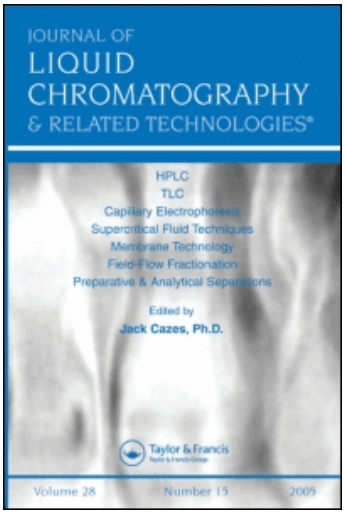

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FRACTIONATION OF PHARMACOLOGICALLY ACTIVE PLANT POLYPHENOLS BY CENTRIFUGAL PARTITION CHROMATOGRAPHY

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ABSTRACT

Centrifugal partition chromatography (CPC) has been applied to the preparative fractionation of pharmacologically active polyphenols contained in the crude extracts from three medicinal plants. 1) (-)-Epigallocatechin gallate, the main component of tea polyphenols, which inhibits tumor promotion, 2) oligomeric hydrolyzable tannins, having host-mediated anti-tumor activity and antiviral activity, contained in *Woodfordia fruticosa* flower, 3) polyphenolic components of licorice, having anti-HIV activity. The results obtained with cartridges of medium volume and of small volume were compared during the fractionation of tea polyphenols.

INTRODUCTION

A large number of compounds, belonging to tannins, flavonoids, and some others, are comprised in the plant polyphenols. The presence of phenolic hydroxyl groups, particularly those adjacent to each other on an aromatic ring, is the structural feature common to these compounds. The formation of a large

molecule composed of such polyphenolic units is another structural feature characteristic of the tannin molecules. Isolation of each polyphenolic compound belonging to tannin in the plant extracts has been retarded until recent years, unlike the other types of natural organic compounds such as alkaloids, terpenoids and steroids. The difficulties in their isolation are mainly due to the properties induced by such structures of tannins. Their instability, which is one of these properties, is enhanced by the presence of ester linkages in the molecules of hydrolyzable tannins. Therefore, the word "tannin" has long been used as if it is a mixture of constituents not isolable, and of composition not reproducible. On the other hand, tannins contained in each plant of different species have often been regarded as the same substance chemically indiscriminate.

The application of recent technics of chromatography facilitated the isolation of polyphenolic compounds of this kind (1). Although some solid supports, such as silicic acid which is widely applicable to the other types of natural organic compounds, are often inadequate for the preparative separation of tannins, several types of gel, Sephadex, Toyopearl, DIAION, and MCI gel, have been found as effective in the separation of tannins (1). Adequate combinations of these gels, solvents and tannins, upon the column chromatography, induced appreciable separation of each component (2-4). However, considerable amount of polyphenolic compounds, especially tannins of large molecule which are strongly adsorbed on these gels, are lost during the column chromatography (5). Hydrolysis of hydrolyzable tannins, particularly of their oligomers, often occurs (6-8).

Application of centrifugal partition chromatography, has much improved the efficiency in the separation of polyphenolic compounds. It can separate compounds without the loss due to adsorption and decomposition on the solid supports, and can be performed in markedly shorter time than droplet counter-current chromatography, thus reducing diffusion in the columns (9).

Several hundreds polyphenols, of the molecular weights up to several thousands, have been isolated from nature, by the aid of recent developments in the chromatographic technics including CPC. Frequently, several technics have been combined to effect better separation and purification of these compounds (9).

Various biological and pharmacological activities, which are often different depending on their chemical structures, have been found for each isolated polyphenol (10). Among these activities are inhibition of tumor promotion (11), host-mediated anti-tumor activity (12), inhibition of HIV (13,14) and other viruses (15), and inhibition of active oxygens (16,17), and several others (18-20).

The preparative fractionation of the polyphenolic compounds by CPC, in the present investigation, was carried out using cartridges of both medium and small volume. Other technics of separation, gel-column chromatography and crystallization, were sometimes combined with the CPC technic, to improve the efficiency in the separation.

The main polyphenols subjected to the isolation in the present work are as follows. a) (-)-Epigallocatechin gallate, the main polyphenolic component in Japanese green tea, which markedly inhibited tumor promotion when applied to skin cancer (11), and also to duodenal cancer orally (21). b) Woodfordin C (22) and oenothein B (23), dimeric hydrolyzable tannins having macro-ring structures, which are contained in the flower of *Woodfordia fruticosa* (Lythraceae), a popular astringent to treat dysentery and sprue, and other diseases in India, Indonesia and Malaysia. These compounds showed the host-mediated anti-tumor and/or antiviral effects (12,14,15,22). c) Polyphenols in Si-pei licorice (root and stolon of *Glycyrrhiza* sp. from the north-western region of China) which is frequently used in the traditional Chinese medicine. Several polyphenols isolated from this licorice showed anti-HIV effect (13).

MATERIALS AND METHODS

Materials

Japanese green tea, the dried leaf after steaming, was prepared from the leaves of *Thea sinensis* cultivated in Shizuoka Prefecture, Japan. This green tea (7.5 g) was extracted with boiling water (110 ml) for 10 min, and after filtration, the resulting aq. solution was lyophylized, to give the aq. extract (2.5 g).

Dried flowers of *Woodfordia fruticosa* were purchased in an Indonesian market. The flowers (1 kg) were homogenized in a mixture of acetone-water (7:3) (10 l x 3), and the concentrated filtrate from the homogenate was extracted with diethyl

ether, ethyl acetate and *n*-butanol, successively. The aq. mother liquor thus obtained was evaporated, to give the aq. extract (106 g).

Si-pei licorice used in this study, was purchased from Tochimoto-tenkaido, Japan. The pulverized licorice (2 kg) was defatted with *n*-hexane (2 l x 3), and then extracted with ethyl acetate (2 l x 3), at room temperature. Evaporation of the solvent gave the ethyl acetate extract (61 g).

Centrifugal Partition Chromatography

CPC Apparatus, (A) Model B-92-N equipped with 12 partition cell cartridges Type 250W (240 ml in total), and (B) Model L-90 containing 12 partition cell cartridges Type 1000E (900 ml in total) (Sanki Engineering, Nagaokakyo, Kyoto) were used. The solvents were pumped into the columns rotating 600 rpm (for the extracts of tea and *W. fruticosa*) or 500 rpm (for the licorice extract) [Apparatus (A)], or 700 rpm [Apparatus (B)], with a pump, Model CPC-LBP-II (Sanki). Flow rate was set at 1 ml/min [Apparatus (A)] or 3 ml/min [Appratus (B)]. Fractions [5 g (A) and 10 g (B)] were collected with a fraction collector, Model SF-160K (Advantec, Tokyo). Eluates were monitored with UV absorbance at 280 nm, and every ten fractions were analyzed by high-performance liquid chromatography (HPLC).

The solvent systems for CPC were as follows: solvent (i), chloroform-methanol-*n*-propanol-water (9:12:2:8, by volume, for tea polyphenols), solvent (ii), *n*-butanol-*n*-propanol-water (4:1:5,*W. fruticosa* tannins), solvent (iii), chloroform-methanol-water (7:13:8, for licorice polyphenols). Distribution coefficients of polyphenols between the upper and lower phases of these solvent systems (at 20°C) were determined by HPLC.

High-Performance Liquid Chromatography

HPLC was performed on a Merck LiChrospher RP-18 (5 μm) column, with acetonitrile-water-acetic acid (2:17:1) (flow rate 1.2 ml/min for tea polyphenols), 0.01M H_3PO_4-0.01M KH_2PO_4-ethanol-ethyl acetate (17:17:4:2) (flow rate 1.1 ml/min for *W. fruticosa* tannins), or acetonitrile-water-acetic acid (8:11:1) (flow rate 1.2 ml/min for licorice polyphenols), at 40°C in an oven. Detection was effected by Shimadzu SPD-6A spectrophotometric detector set at 280 nm.

TABLE 1

Distribution Coefficients of Polyphenols between Upper and Lower Phases

Compound	Distribution coefficient at 20°C (upper phase/lower phase)
Tea constituents [solvent system (i)]	
(-)-Epigallocatechin gallate	7.3
(-)-Epigallocatechin	6.9
(-)-Epicatechin gallate	3.9
(-)-Epicatechin	2.9
Caffeine	0.27
Tannins of *W. fruticosa* [solvent system (ii)]	
Woodfordin C	0.36
Oenothein B	0.19
Licorice polyphenols [solvent system (iii)]	
Licopyranocoumarin	0.37
Glycycoumarin	0.22
Glycyrrhisoflavone	0.15

RESULTS AND DISCUSSION

Distribution coefficients of the polyphenols between the upper and lower phases of the solvent systems used are summarized in Table 1.

(I) (-)-Epigallocatechin Gallate and Other Tea Polyphenols

The distribution coefficients of four tea polyphenols between the upper phase and lower phase of solvent system (i) were all over 1, and were significantly different from each other as shown in Table 1. Good separation was therefore expected by the application of the lower organic phase as the mobile phase of CPC.

In the initial experiment, relatively small amount of the aq. extract (1 g) of green tea leaves was subjected to CPC using cartridges of smaller volume [Apparatus

(A)], developing in the "descending mode" with solvent system (i) to separate the tea polyphenols. The result is shown in Figure 1. The major three peaks of the elution curve are those of caffeine, (-)-epicatechin gallate and (-)-epigallocatechin gallate. The HPLC profiles for several combined fractions in the figure show the purity of four polyphenols and caffeine obtained in this way.

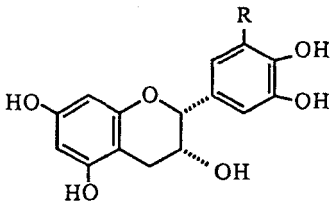
Then, a larger amount (10 g) of the aq. extract of green tea was subjected to CPC using Apparatus (B), with the same solvent system. As shown in Figure 2, the result is similar to that of the initial experiment, and no marked decrease of efficiency in the separation due to the increase of the sample is observed. This single operation yielded 1 g of (-)-epigallocatechin gallate of the purity >80%, which gave pure compound when crystallized once from water.

(II) Tannins of *Woodfordia fruticosa*

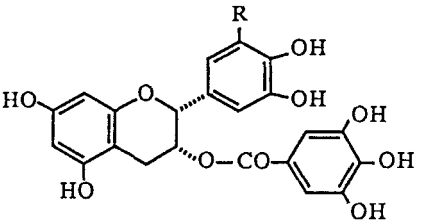
Among over ten hydrolyzable tannins contained in *W. fruticosa* (22), main dimeric hydrolyzable tannins in the aq. extract were oenothein B and woodfordin C. Structural difference of these two is only in the presence or absence of a galloyl group on one of the two anomeric hydroxyl groups. However, the distribution coefficients of these two tannins, between upper and lower phases of solvent system (ii), which had been successfully applied to the separation of various types of tannins, showed appreciable difference. These coefficients also suggested that good separation is expectable when the upper organic phase is used as the mobile phase.

The aq. extract (1 g) of *W. fruticosa* was then subjected to CPC [Apparatus (A)] in the "ascending mode" with solvent system (ii). The HPLC profiles in Figure 3 shows that woodfordin C and oenothein B were satisfactorily separated from each other, in spite of broadening of the region containing oenothein B in the elution curve. This broadening may be due to the equilibration (24) induced by the anomerization in the two glucopyranose cores.

It is noteworthy that these dimers were free of non-tannin materials, such as sugars, amino acids, proteins and inorganic materials, which remained in the stationary phase in large amount after the development. The removal of such non-tannin substances, in addition to shortening of the long developing time required



(-)-Epicatechin (EC): R=H
(-)-Epigallocatechin (EGC): R=OH



(-)-Epicatechin gallate (ECG): R=H
(-)-Epigallocatechin gallate (EGCG): R=OH

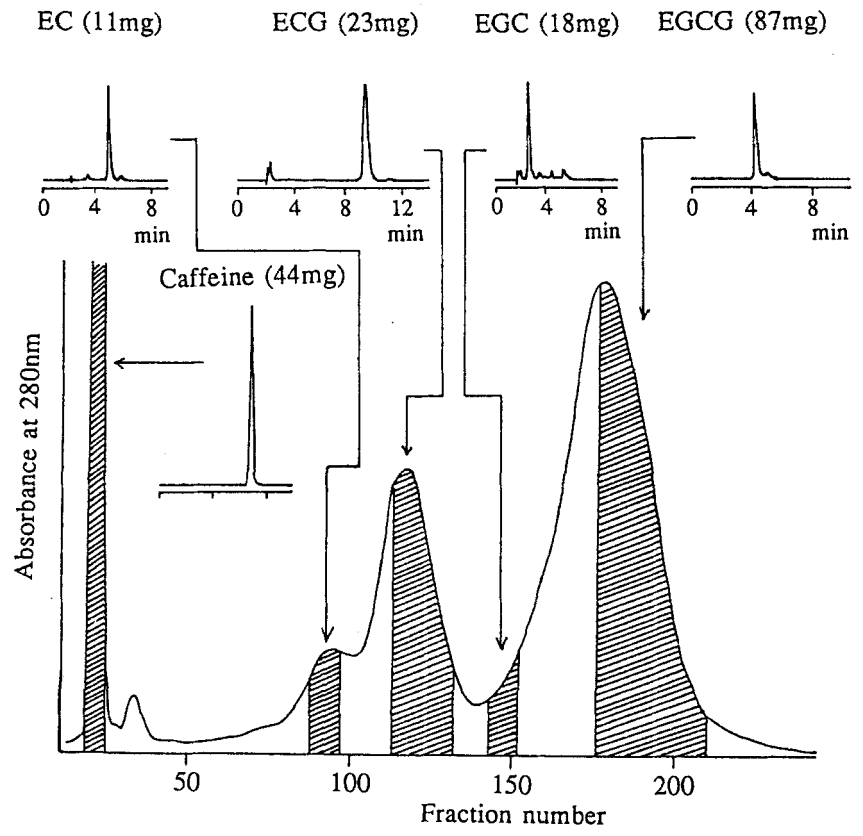


FIGURE 1 CPC of tea extract (1 g) with cartridges Type 250W, and HPLC profiles of combined fractions.

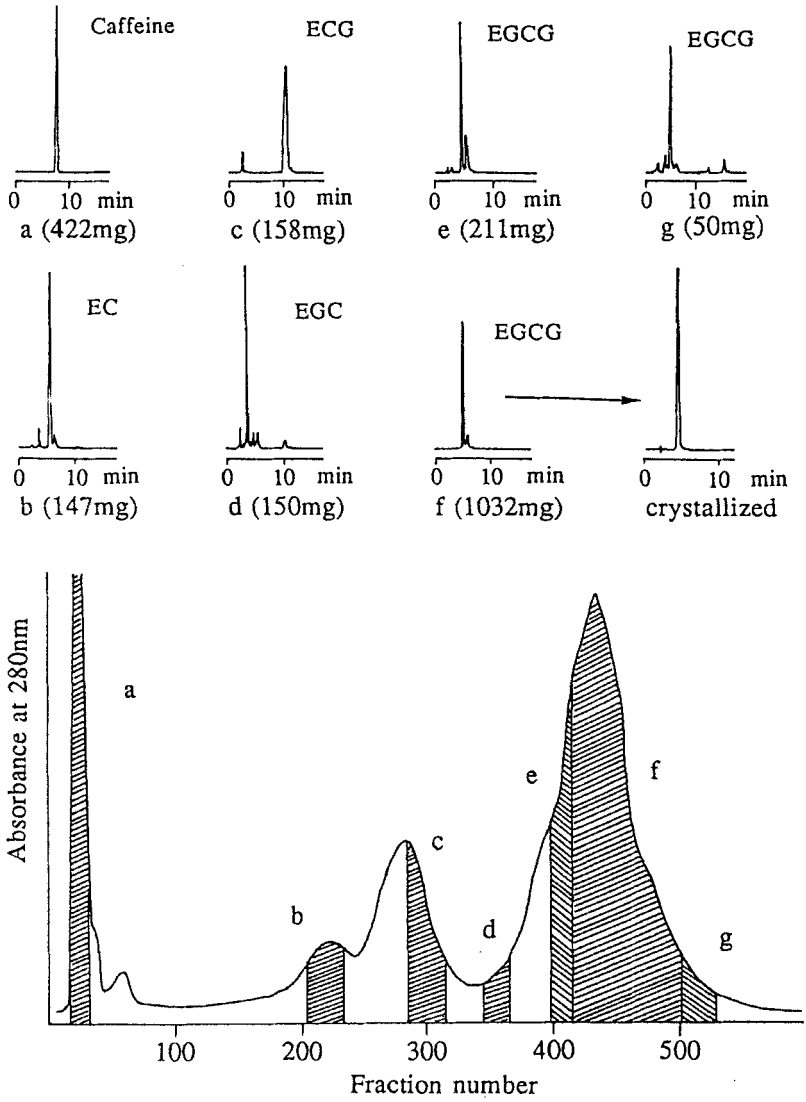


FIGURE 2 CPC of tea extract (10 g) with cartridges Type 1000E, and HPLC profiles of combined fractions.
(-)-Epigallocatechin gallate was crystallized from water.

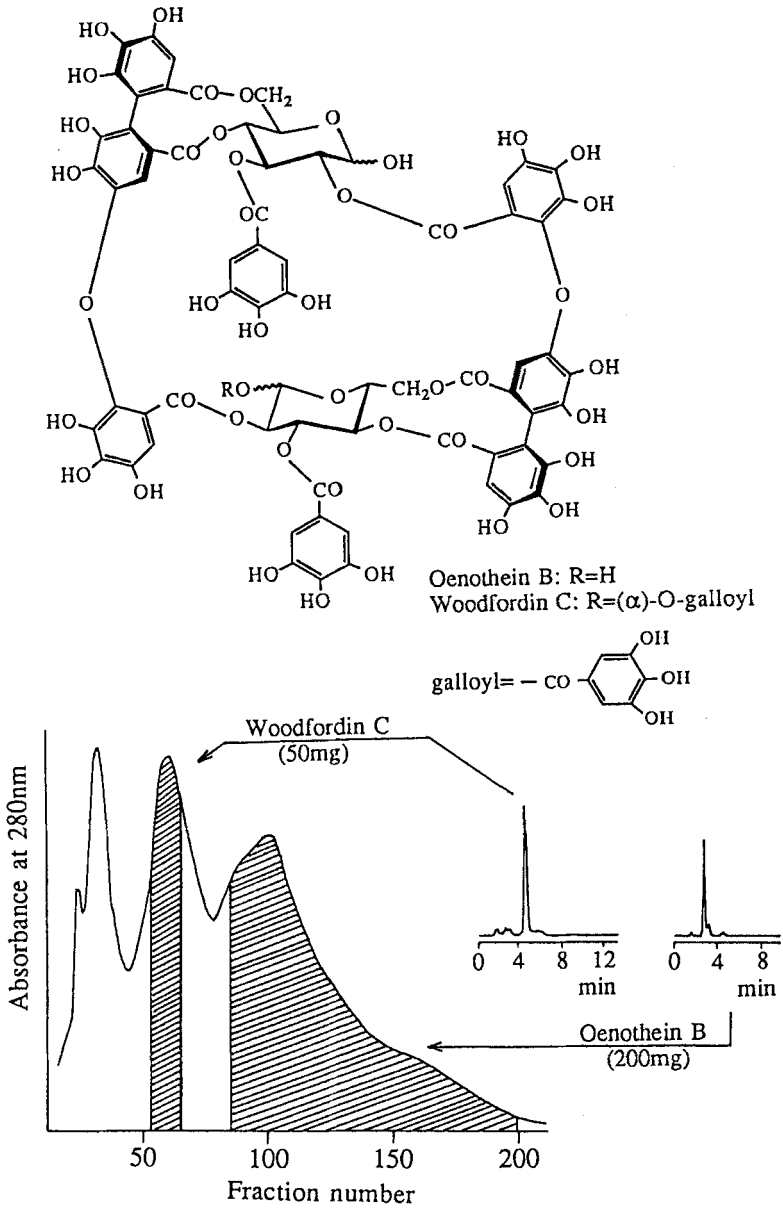


FIGURE 3 CPC of tannins in aqueous layer from *Woodfordia fruticosa*, and HPLC profiles of combined fractions.

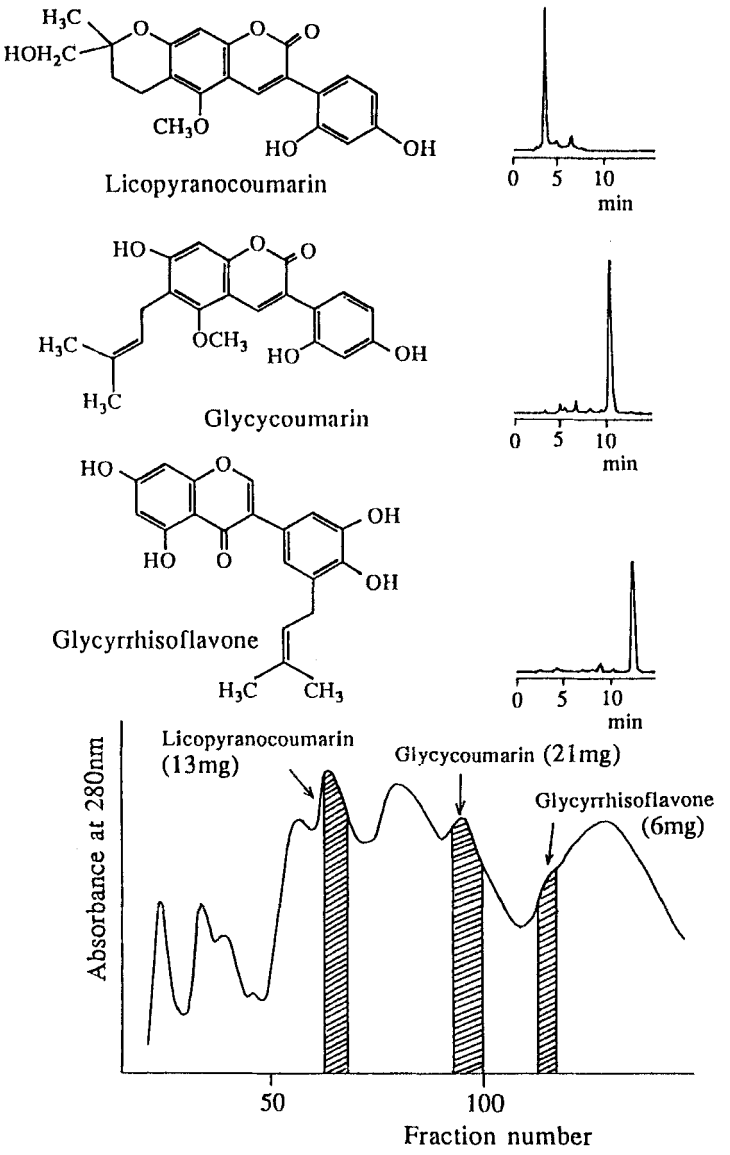


FIGURE 4 CPC of ethyl acetate extract of Si-pei licorice, and HPLC profiles of combined fractions.

for column chromatography, is an advantage of combining CPC with column chromatography.

(III) Licorice Polyphenols

Recent investigation revealed that licorice contains, besides glycyrrhizin, a large variety of flavonoids, which are considerably different among several kinds of licorice from different origins (25). Such difference has been also found in the structures of flavonoids we isolated from Sinkiang licorice and Si-pei licorice (13,26). We previously reported that CPC is applicable to separation of the components of the extract from Sinkiang licorice (6), whereas column chromatography of this extract on silica gel causes irreversible adsorption of such phenolic compounds. Now we have applied CPC to the isolation of three bioactive flavonoids from a mixture of a large number of phenolic compounds in Si-pei licorice.

The upper aq. phase of solvent system (iii) was used as the mobile phase ("ascending mode"), based on the distribution coefficients in Table 1. The result obtained by the fractionation of the ethyl acetate extract (1 g) of Si-pei licorice, and the HPLC profiles are shown in Figure 4. Each flavonoid thus obtained was further purified by column chromatography on MCI gel CHP-20P. Fractionation of the same extract in a larger scale (30 g) was performed using the 1000E cartridges [Apparatus (B)], with little lowering of the efficiency in the separation.

Our fractionation experiments of the polyphenol mixtures performed by the present time show that the separation by CPC does not always lead to the final purification in the preparative scale, and preparative HPLC often effects better separation as far as samples are not lost extensively by adsorption. However, the applicability of CPC to the separation of fairly large amount of samples (10-30 g or more) such as tannins, without their loss, is a great advantage of CPC to the other methods, including preparative HPLC.

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