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Synthesis and characterization of water-soluble chitosan derivate and its antibacterial activity

Yajun Xie, Xiaofei Liu *, Qiang Chen

School of Material Science and Engineering, Tianjin University, Tianjin 300072, China

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Abstract

Ethylamine hydroxyethyl chitosan (EHCs) was synthesized from chitosan and chloroethylamine hydrochloride under alkali condition by the novel method described in this study. The effect of several factors, such as reaction time, temperature and the molar ratio of hydroxyethylic chitosan (HECs) to chloroethylamine hydrochloride were discussed. The structure changes of derivative were investigated by FTIR, ^{13}C NMR, DSC and TGA analysis. Good water solubility of EHCs was observed in a wide range of molecular weights. Antibacterial activities of derivative against *Escherichia coli* (*E. coli*) was explored by the optical density method.

Keywords: Antibacterial activity; Chitosan derivate; Ethylamine Hydroxyethyl chitosan; Quaterisation; Water solubility

1. Introduction

Chitosan, a copolymer of glucosamine and *N*-acetylglucosamine units linked by 1–4 glucosidic bonds, is obtained by *N*-deacetylation of chitin, which is the second most naturally occurring biopolymer (after cellulose) (Bartnicki-Garcia, 1968). Chitosan is a biocampatibale polymer reported to exhibit a great variety of useful biological properities such as anticholesteremic (Muzzarelli, 1996) and ionsequestering actions (Peter, 1995). Recently, the antibacterial and antifungal activities of chitosan have been followed with great interest. Chitosan inhibits the growth of a wide variety of bacteria and fungi (Chun, Jang, Heung, & Kyu, 1997; Fang, Li, & Shin, 1994; Hiroshi, 1993; Liu, Guan, & Yao, 2001; Seo, Mitsuhashi, & Tanibe, 1991; Uchida, 1988; Uchida, Izume, & Ohtakara, 1988; Yalpani, Jhonson, & Robinson, 1991), showing broad spectra of antibacterial activity, high killing rate and low toxicity toward mammalian cells (Franklin & Snow, 1981; Takemono, Sunamoto, & Akasi, 1989). However, despite this desirable characteristic, its actual use is limited because of its poor solubility in water. Chemical modification is helpful in improving the water solubility of chitosan and its derivatives, thus widening their applications (Alexandrova, Obukhova, Domnina, & Topchiev, 1999; Sashiwa & Shigemasa, 1999; Sridhari & Dutta, 2000; Sugimoto, Morimoto, Sashiwa, Saimoto, & Shigemasa, 1998; Terada et al., 1999). Thereinto quaternary ammonium chitosan is prepared by introducing quaternary ammonium group on dissociative hydroxylgroup or amino group, prevenient study found that after quarternization, derivates exhibited better water solubility and stronger antibacterial activity than chitosan. since most of the unique properties of chitosan are attributable to its cationic nature. Quaternary chitosan derivatives have various potential pharmaceutical applications, e.g., as permeation enhancers (van der Merwe, Verhoef, Verheijjden, Kotze, & Junginger, 2004), as gene delivery systems (Kean, Roth, & Thanou, 2005) and as antimicrobials (Lim & Hudson, 2004), and also some other possible applications, e.g., in paper making (Li, Wu, & Zhan, 2004).

We report here a novel synthetic procedures for the preparation of chitosan derivative with a quaternary amino

* Corresponding author. Tel.: +86 22 27408099; fax: +86 22 23550646.
E-mail address: Liuxf@tju.edu.cn (X. Liu).

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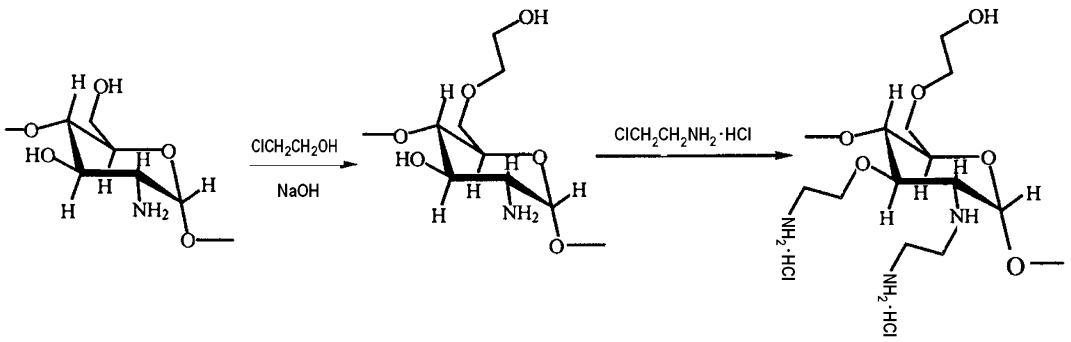

Fig. 1. The synthesis of ethylamine hydroxyethyl chitosan (EHCs).

salt. This quaternary derivative (Fig. 1) was prepared by treating hydroxyethyl chitosan (HECs) with chloroethylamine hydrochloride in sodium hydroxide solution, expecting to improve its water solubility and antibacterial activity further. Derivate was characterized by Fourier transform infrared spectroscopy (FTIR), differential scanning calorimetry (DSC), ^{13}C NMR, The solubility in H_2O were also studied as well as the antibacterial activity against *Escherchia coli* (*E. coli*) which was explored by the optical density method.

2. Experimental

2.1. Materials

Chitosan (molecular weight 1.08×10^6; the degree of deacetylation 0.85), a commercial material supplied by Qingdao Medicine Institute, was depolymerized via γ irradiation degradation to a series of low molecular weight samples. All other reagents are analytical grade provided by The 3rd chemical reagent factory of Tianjin, China.

Escherichia coli supplied by Degradation Laboratory of Nankai University, Tianjin, China.

2.2. Preparation

The process is divided into two steps:

(1) Five grams of chitosan was added into a certain number of sodium hydroxide solution(50%,by weight) with stirring, then the mixture was put into refrigerator at −18 °C for 48 h for alkalization. After thawing, Alkali chitosan and Isopropyl alcohol(10 ml) were mixed and stirred to homogenization, then some chloroethanol was added with continuous stirring at 80 °C for 12 h. After filtration, the solid product was washed with alcohol for two times. HECs yielded was dried in an oven at 60 °C.
(2) Five grams of HECs was poured into a mixture of 20 ml Isopropyl alcohol and 42% sodium hydroxide solution with stirring, the reaction was react at certain temperature for some time after some chloroethylamine hydrochloride was added in. When the reaction ended, adjust PH to 7 using 1 mol/l hydrochloric acid solution, the solid product was washed with acetone for several times and dried in an oven.

The optimal conditions of preparing EHCs ($M_n = 1.9 \times 10^5$) were determined on the basis of orthogonal tests. NH_2% of EHCs was tested by Potentiometric titration.

2.3. Characterization

FTIR spectra of EHCs was obtained with a Nicolet 560 ESP spectrometer. After being dried completely at 50 °C, the samples could be used for FTIR analysis with ordinary KBr pellet method.

^{13}C NMR was obtained with a Mercury-Vx300 spectrometer. Chitosan derivate were dissolved in D_2O containing tetramethylsilane (TMS) as an internal standard.

The results of DSC and TG were obtained with Perkin–Elmer DSC7 and Perkin–Elmer TGA7 equipment respectively. The temperature range is 30–550 °C and heating rate of 10 °C/min.

2.4. Solubility

Ten gram samples were got and dispersed in 90 g H_2O, then stirred at 90 °C for 12 h. Undissolved section was separated and washed with acetone, then dried in an oven under a vacuum. We defined solubility (Sa):

$$\mathrm{Sa} = [(10 - W_1)/10] \times 100\%. \quad (1)$$

W_1: the weight of undissolved section (g)

Experiment was carried out three times and draw average number.

2.5. Antibacterial assessment

Antibacterial activity of EHCs against *E. coli* was evaluated by using optical density method described as follows: A representative bacteria colony was picked off, placed in a nutrient broth (peptone 10 g, beef extract 3 g, NaCl 5 g in distilled water 1000 ml; pH 7.0–7.2) and incubated at 37 °C for 24 h. Then the obtained fresh culture where

bacteria cells grew luxuriantly was ready for antibacterial test. 0.2mL of the fresh culture was inoculated to the medium (9.8 mL) containing chitosan derivatives and incubated in a shaking bed (150 rpm) at 37 °C for 24 h. During incubation, turbidity of the medium was measured at 610 nm for six times with an UV spectrophotometer (Unico UV-2000, Shanghai, China).

3. Result and discussion

3.1. Preparation of EHCs

The optimal condition for preparing EHCs was studied by the orthogonal test. Three controllable variables, reaction time (h), temperature and the molar ratio of HECs to chloroethylamine hydrochloride, were selected, each at three levels.The investigated variables and their test levels are listed in Table 1. The test results are listed in Table 2. As results indicated, in our study range, the order of influence of each variable on the Mass is C1 > A1 > B1, while A2 > C2 > B2 on NH_2%. Thus the optimum reaction conditions were determined as follows: Temperature 70–80 °C; the molar ratio of HECs to chloroethyl amine hydrochloride, 2:1 and the reaction time, 12 h. The experiment showed that appropriate reaction condition was necessary to prepare EHCs.

3.2. FTIR analysis

FTIR spectra of the HECs and EHCs is showed in Fig. 2, from spectra of HECs we see, Peaks at 1031 cm^{-1} assigned to stretching vibration of –CO(H) at C_6 shifted to a new positon and became sharper compared to that of chitosan, peaks at 1650 cm^{-1} and 897 cm^{-1} were ascribed to absorbtion of primary amine, the existence of which and there was no absorbtion of secondary amine proved that reaction did react at C_6–OH while not C_3–OH or –NH_2.

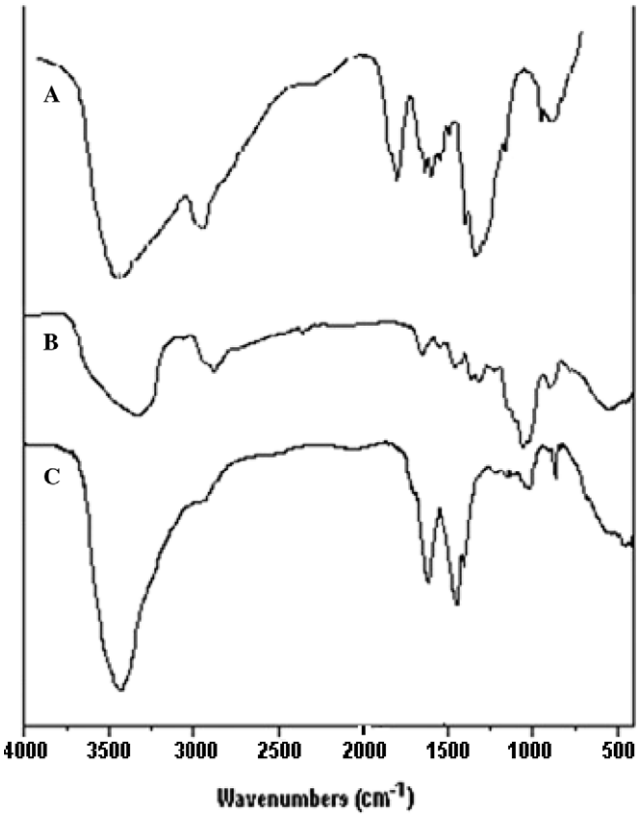

Fig. 2. FTIR spectra of chitosan (A), HECs (B) and EHCs (C).

For EHCs, intensified peak at 1618 cm^{-1} were ascribed to transmutation vibration of $-NH_2$ owing to a large quantity of NH_2 group was fetched in. Peaks at 1448 cm^{-1} and 1409 cm^{-1} were assigned to transmutation vibration and swing vibration of –CH_2 on –CH_2–N-, respectively, it also demonstrated that $-NH_3^+$ group was fetched in. After quaterisation, the absorbtion corresponding to hydroxyl group at 1058 cm^{-1} disappeared. Peaks at 1022 cm^{-1} characters stretching vibration of secondary

Table 1
The orthogonal tests details ($M_n = 1.9 \times 10^5$)

Experiment	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	No. 7	No. 8	No. 9
Ratio	1:1	1:1	1:1	1:2	1:2	1:2	1:3	1:3	1:3
Time (h)	6	12	24	6	12	24	6	12	24
Temperature (°C)	50	70	80	70	80	50	80	50	70
Mass (g)	5.21	5.75	6.01	5.91	6.68	5.86	5.98	5.75	6.58
NH_2%	8.40	9.51	9.85	10.31	10.70	9.36	9.85	10.5	10.2

Table 2
The results of the orthogonal tests

	Analysis based on mass				Analysis based on NH_2%		
	Ratio (A1)	Time (B1)	Temperature (C)		Ratio (A2)	Time (B2)	Temperature (C2)
R1	5.657	5.700	5.607	R1′	9.253	9.420	9.517
R2	6.150	6.060	6.080	R2′	10.120	10.003	10.267
R3	6.103	6.150	6.223	R3′	10.183	10.133	9.803
R	0.493	0.450	0.616	R	0.930	0.713	0.720

amine group, illuminated that reaction of amination did reacted at $–NH_2$ simultaneity. Because a large quantity of secondary amine group was fetched in, stretching vibration peak at 867 cm^{-1} which shifted to a lower frequency compared to chitosan owing to the affect of hydrogen bond was intensified.

3.3. ^{13}C NMR analysis

The ^{13}C NMR spectrum of EHCs is presented in Fig. 3, the pyranoid ring protons (C-1,2,3,4,5,6) were considered to resonate at 97.6, 56.7, 70.1, 76.7, 74.0, 60.1 ppm, respectively, peak at 83.9 ppm was assigned to $–CH_2NH_2$, peak at 48.2 ppm was ascribed to $–CH_2–O–$ while peak at 22.2 ppm represented the existence of a little of CH_3 group.

3.4. DSC analysis

DSC thermograms of chitosan and EHCs are shown in Fig. 4. The spectra of chitosan shows a broad endothermic peak around 103.9 °C and sharp exothermic peak at 322.6 °C. The former endothermic peak may be due to the water vapor that the chitosan contains. While the latter may be attributed to the decomposition of chitosan. The endothermic peak of EHCs around 96.31 °C may be due to the loss of water and moisture content in the polysaccharide. The exothermic peak at 229.69 °C corresponds to its thermal decomposition. The results indicated that the structure of chitosan chains has been changed due to the introduction of a large quantity of –OH and $–NH_2$ and the reduced ability of crystalization.

Thermographs of the chitosan and EHCs are shown in Fig. 5. The chitosan shows slow weight loss starting from 140.0 °C to 200.0 °C due to the decomposition of polymer with low molecular weight, followed by more obvious loss of weight starting from 200 °C to 310 °C, which could be attributed to a complex process including dehydration of the saccharide rings, depolymerization and decomposition of the acetylated and deacetylated units of the polymer (Peniche & SanRoman Arguelles-Monal, 1993). A fast process of weight loss appears in TG curve for EHCs decomposing from 170 °C to 300 °C. The results demonstrate the loss of the thermal stability for EHCs to the original chitosan. Introduction of –OH and $–NH_2$ group into polysaccharide structure should disrupt the crystalline structure of chitosan, especially through the loss of the hydrogen bonding.

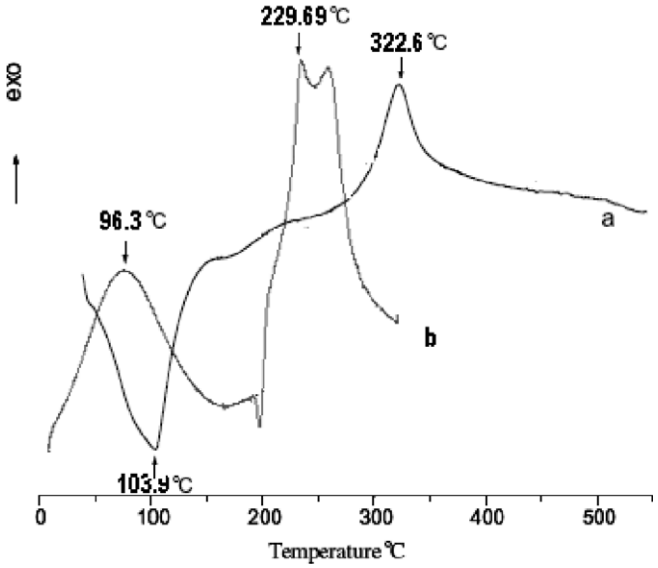

Fig. 4. DSC curves of chitosan (a) and EHCs (b).

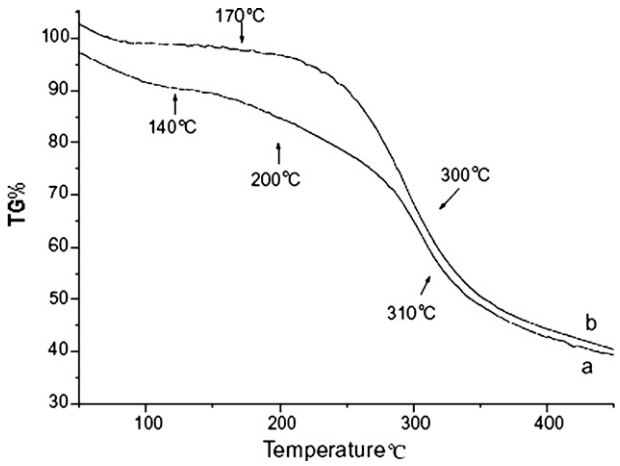

Fig. 5. TG curves of chitosan (a) and EHCs (b).

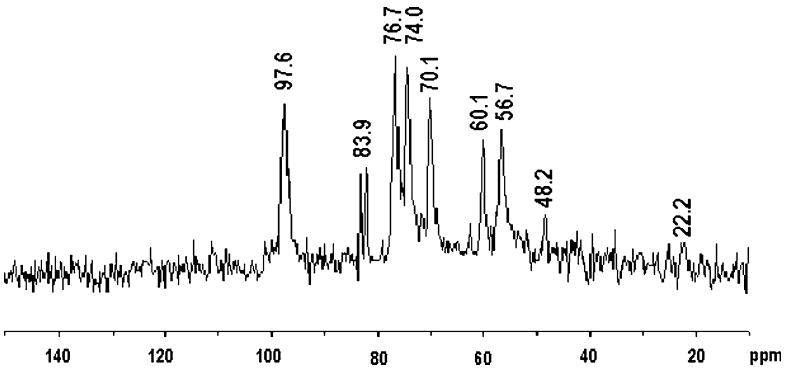

Fig. 3. ^{13}C NMR spectrum of EHCs.

3.5. Solubility

Table 3 shows the solubility of EHCs synthesized with different reaction conditions determined on the basis of orthogonal tests. The results show that all derivates with $M_n = 1.9 \times 10^5$ can dissolve in H_2O except NO.1 condition, especiallly the solubility of NO.5 reached 63.5 mg/ml. It suggested that EHCs is a good water-soluble chitosan derivate, EHCs decreased the intermolecular interactions, such as van der Waals forces, and then increased water solubility.

Table 4 shows the solubility of different molecular weight of HECs and EHCs, thereinto EHCs were synthesized under NO.5 condition. The EHCs can dissolve in all molecular weight range but HECs can dissolve in H_2O only when M_n below 8×10^4. The results show that EHCs exhibited better water solubility than HECs with the increase of molecular weights, The higher water solubility of EHCs is attributed to the decrease of intermolecular interactions, such as van der Waals forces; the lower the molecular weight, the lower the intermolecular attraction forces (Kubota, Tatsumoto, Sano, & Toya, 2000). Therefore, the decreasing in water solubility of the EHCs with high molecular weight is probably due to the high molecular weight itself.

3.6. Antibacterial assessment

The antibacterial activity of EHCs was explored by using optical density method. The *E. coli* bacteria were selected as test cells because they are the most frequent bacteria in wound infection and representative Gram negative bacteria. Fig. 6 shows OD at 610 nm versus exposure time for EHCs with *E. coli*, which EHCs ($M_n = 1.9 \times 10^5$) was synthesized with different reaction conditions determined on the basis of orthogonal tests. As shown in the figure, the values of OD of derivates are are much less than blank analysis and are stable with the increasing of time, which shows that this chitosan derivative has a high rate of killing cells and high antibacterial activity. After 2 h the cell survivors decline slowly or have a small increase, especially NH_2% at 7.42%. This means that EHCs has low bacteriostatic action at low NH_2%.

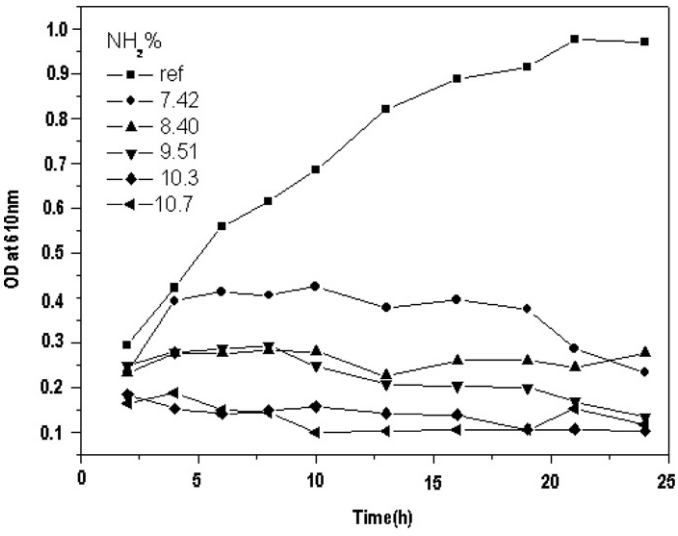

Fig. 6. NH_2% dependence on the antibacterial activity of EHC ($M_n = 1.9 \times 10^5$).

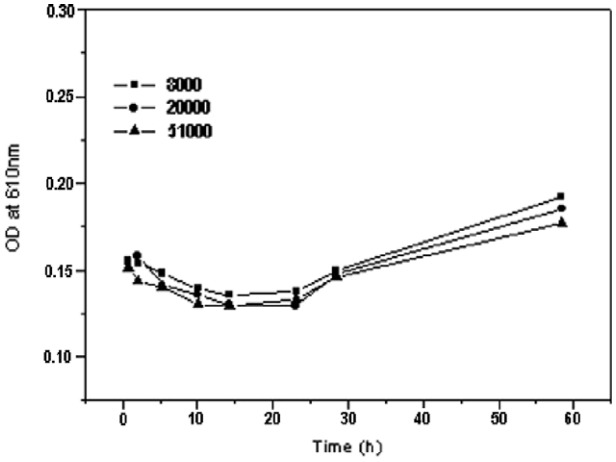

Fig. 7. The molecular weight dependence on the antibacterial activity of EHCs.

Fig. 7 shows the effect of EHCs against *E. coli*, which EHCs was synthesized with different molecular weights. More than 80% of *E. coli* were killed within 2 h, After that the cell survivors decline slowly within 24 h and have a

Table 3
The solubility of EHCs in H_2O ($M_n = 1.9 \times 10^5$)

Experiment	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	No. 7	No. 8	No. 9
Sa (mg/ml)	—	12.3	40.8	14.6	63.5	35.7	53.1	25.3	60.1

Table 4
The influence of M_n of HECs and EHCs solving in H_2O

M_n		8×10^3	2×10^4	5.1×10^4	8×10^4	1×10^5	1.9×10^5	2.7×10^5
Solubility	HECs	√	√	√	√	×	×	×
	EHCs	√	√	√	√	√	√	√
Sa(mg/ml)	HECs	90.3	57.6	34.9	23.2	—	—	—
	EHCs	102.1	72.3	70.9	65.1	64.2	63.5	42.5

√: soluble, ×: insoluble.

small increase in the next 36 h. Thereinto, EHCs showed better antibacterial activity when the molecular weights above 5.1×10^4.

4. Conclusion

Ethylamine hydroxyethyl chitosan (EHCs) with good water solubility was prepared via a novel processing technique, the optimum reaction conditions were determined as follows: temperature 70–80 °C; the molar ratio of HECs to chloroethylamine hydrochloricde, 2:1 and reaction time, 12 h. FTIR and 13C NMR confirmed that the quaternary amino salt group was introduced onto chitosan. The paper suggested that EHCs decreased the intermolecular interactions, such as van der Waals forces, and then increased water solubility. Derivate showed good inhibition effects against *E. coli*, while the factor which affected the antibacterial activity most was NH_2%.

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