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Comparison of the properties of dextran and hydroxyethyl starch substituted with benzene tetracarboxylate in terms of their use in blood transfusion

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Solutions of dextran and hydroxyethyl starch are used as plasma substitutes but are not capable of carrying oxygen *in vivo*. To transform these solutions into blood substitutes, it has been suggested that the natural oxygen-carrier protein, i.e. human haemoglobin, is bound to these polymers. However, the polymers have to be modified so that in the protein conjugate haemoglobin exhibits the appropriate oxygen-binding properties. Thus, covalent conjugates of oxyhaemoglobin and of dextran substituted with benzene tetracarboxylate have been used and appear effective vascular oxygen carriers. The same procedure was applied to hydroxyethyl starch but this polysaccharide, because of its branched nature, could not be substituted with benzene tetracarboxylate without being highly cross-linked as evidenced by NMR and low-angle laser light-scattering analyses. As a consequence, in the haemoglobin–hydroxyethyl starch covalent conjugates, the polymer-linked benzene tetracarboxylate groups are not easily accessible to the allosteric site of the protein as in the dextran conjugates, and therefore cannot improve its oxygen-binding properties.

INTRODUCTION

Dextran, a polysaccharide which is biodegradable in humans, has been used as a plasma expander for a long time. It is well tolerated, non-toxic and relatively non-antigenic, provided its molecular weight is not too high ($\overline{M}_w < 70\,000$) (Gruber, 1976; Thorén, 1978). Recently, the hydroxylated derivative of another polysaccharide, starch, has shown great potential in this field since it is even better tolerated than dextran, whatever the molecular weight (Maurer, 1968; Thomson, 1978; Moser, 1986). Hydroxyethyl starch (HES) is obtained by the reaction of ethylene oxide on acid-hydrolysed waxy maize starch (Moser, 1986). This derivatization has two advantages: first, it improves the solubility of the polysaccharide in water and second, it provides it with an in-vivo protection against the hydrolytic action of α-amylase, thus giving it a plasmatic half-life which is long enough to enable it to be used as a plasma expander (Thomson, 1978; Mishler, 1980).

Among the different methods used to prepare blood substitutes, one approach consists of transforming polymeric plasma expanders into oxygen carriers by binding them to human haemoglobin (Hb). This covalent association leads to a significant increase in the intravascular persistence of the protein since it can no longer diffuse through biological membranes, or dissociate into dimers. However, Hb isolated from red cells exhibits too high an oxygen affinity due to the lack of its natural polyanionic effector, 2,3-diphosphoglycerate; it is therefore necessary to irreversibly link to the polymer–protein conjugate, a molecule capable of playing the same role as the natural effector. This we achieved by linking Hb to derivatives of dextran bearing benzene polycarboxylates (Sacco *et al.*, 1989; Dellacherie, 1990; Dellacherie & Vigneron, 1991; Dellacherie *et al.*, 1992). The products have a long intravascular half-time and oxygen-binding properties

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compatible with physiological needs and are now the object of an extensive study on animals (Fasan *et al.*, 1992; Faivre *et al.*, 1992).

Nevertheless as anaphylactoid shock has been reported occasionally with dextrans even of low molecular weights (Laxenaire *et al.*, 1976), we studied the possibility of using HES instead of dextran and applied the same derivatization procedures in order to link the resulting derivatives to Hb. This paper describes the results obtained in the functionalization of dextran and HES with benzene tetracarboxylate, and compares the oxygen-binding properties of their respective conjugates with Hb.

MATERIALS AND METHODS

Materials and chemicals

Dextran T10 ($\overline{M_w} \simeq 11\,000$) was purchased by Pharmacia (Sweden). HES was kindly supplied by Pfeifer & Langen (Germany). Its molecular weight, $\overline{M_w}$, and its average molecular substitution, reported by the supplier, were 40 000 and 0·5 mol of hydroxyethyl group per mol of glucose unit respectively. Benzene 1,2,4,5-tetracarboxylic anhydride (BTCA) was obtained from Aldrich (Belgium) and 3-ethyl-1-(3-dimethyl-aminopropyl) carbodiimide hydrochloride (EDCI) from Fluka (Switzerland). The purified Hb solutions (10%) were prepared at the Centre Regional de Transfusion Sanguine (Nancy, France) according to the classical method (Labrude *et al.*, 1988).

Synthesis of modified polysaccharides (Dex-BTC and HES-BTC)

Dextran or HES (1 g) was dissolved in water (from 1 ml to 40 ml depending on the experiment) and the pH raised to 9·2 with 1 M NaOH. The desired amount of BTCA was slowly added to the solution while keeping the pH at 9·2 by simultaneously adding 2 M NaOH. The mixture was allowed to react during the night at ambient temperature. The remaining benzene tetracarboxylic acid was removed by chromatography on a 50 × 2·5 cm column of AcA 202 Ultrogel (IBF, France) equilibrated with 0·05 M phosphate buffer, pH 7·2. The polysaccharide-containing fraction was then dialysed against water and freeze-dried.

Synthesis of the conjugates of Dex-BTC or HES-BTC with oxyHb

The desired amount of polymer was dissolved in water (10 ml) and 15 ml of a 10% Hb solution was added (5°C). The solution pH was then adjusted to 6·5 and the desired amount of EDCI was added. The mixture was allowed to react at 5°C for 2 h, dialysed against water and then stored at −20°C.

Methods

The BTC content of the polysaccharides was determined by UV spectroscopy in 0·01 M NaOH ($\varepsilon = 2{\cdot}44 \times 10^4$ $mol^{-1}\,cm^{-1}$, $\lambda_{max} = 213$ nm). ^{13}C-NMR spectra were run in D_2O on a 200 MHz Bruker AM spectrometer with sodium 3-(trimethylsilyl)-1-propane sulphate as an internal reference. The polymer concentration was between 400 and 600 mg ml^{-1} depending on the BTC content. A relaxation time of 14 s was determined as the optimum.

The $\overline{M_w}$ values of polymers were determined by static LALLS (low-angle laser light scattering) in 0·1 M NaCl solutions using a Chromatix KMX6 (Milton Roy, LDC, Riviera Beach, USA). The distribution of molecular weights was studied for some samples by the SEC-LALLS method (size exclusion chromatography coupled to the LALLS detector) at 35°C using a Waters-Millipore HPLC apparatus equipped with the Chromatix KMX6 and a refractometric detector (Waters-Millipore 410). The SEC columns (Ultrahydrogel 500 + Ultrahydrogel 1000, Waters-Millipore) were used with 0·1 M NaCl as eluent and a flow rate of 0·7 ml min^{-1}, and 20 µl of polymer solutions (5–10 mg ml^{-1}) were injected. The dn/dc values were determined at 35°C in 0·1 M NaCl with a Brice-Phoenix differential refractometer equipped with a 633-nm laser light source.

High-performance gel chromatography of polymer–Hb conjugates was carried out on a 0·75 × 30 cm TSK G4000 SW column (from Touzart & Matignon, France) with 0·05 M phosphate buffer pH 7·2 as eluent, at a flow rate of 0·7 ml min^{-1}.

Oxygen-binding curves were determined by the spectrophotometric method, using a tonometer, in 0·05 M Tris–HCl buffer, pH 7·2, at 25°C as previously described (Dellacherie *et al.*, 1983).

RESULTS

Synthesis and properties of Dex-BTC and HES-BTC

Dex-BTC and HES-BTC were synthesized according to the scheme shown in Fig. 1. Table 1 shows that the reactivity of the two polysaccharides with BTCA is similar though HES is slightly less substituted than dextran. The $\overline{M_w}$ values of the derivatives were determined by static LALLS and the results are shown in Table 2. In this table, the theoretical $\overline{M_w}$ values are also given. The latter were calculated assuming that the substitution was statistically distributed along the polymer chains and that there was no intermolecular cross-linking, or depolymerization. The $\overline{M_w}$ values of the unsubstituted dextran and HES, determined by LALLS, were 11 500 and 28 300 respectively.

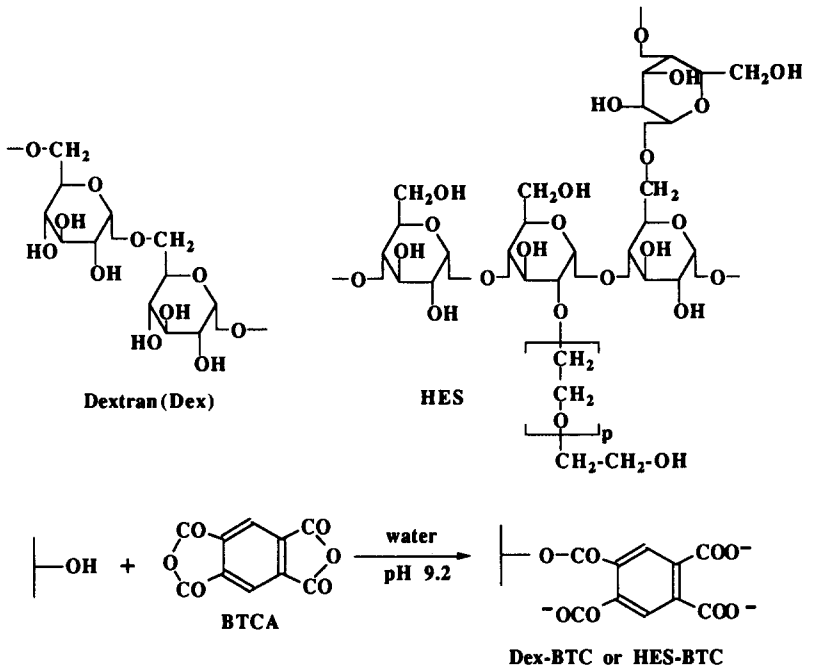

Fig. 1. Linkage of BTCA with the polysaccharides.

Table 1. Comparison of the reactivities of dextran and HES towards BTCA

	Dextran		HES	
	D_1	D_2		
r^a (mmol g^{-1})	0·6	6	0·7	6
r'^b (mmol g^{-1})	0·35	1·25	0·35	0·9

[a] r = BTCA/polymer ratio used for the reaction.
[b] r' = BTC content of the modified polysaccharides.

The polymer samples were examined by ^{13}C-NMR and two characteristic spectra are given in Fig. 2. Figure 2(a) shows the spectrum of Dex-BTC D_2 (Table 2). Peaks are observed around 178 ppm, characteristic of the carboxylate groups of dextran-linked BTC and around 171 ppm corresponding to the ester —COO— groups. In addition to the determination of the BTC content, which, in fact, turned out to be similar to that determined by UV spectroscopy (within ±5%), the spectra allowed us to estimate the percentage of BTC groups linked to the dextran chains by two ester functions, i.e. leading to intra- or intermolecularly cross-linked polymeric chains as represented in Fig. 3. These values, given in Table 2, were calculated from the areas of the peaks corresponding respectively to the carboxylate (178 ppm) and ester (171 ppm) functions. In fact, if the BTC groups are linked to the dextran chains by only one ester function, the area of the —COO$^-$ carboxylate peaks will be 3 times larger than the —COO— ester peaks. If some BTC groups are linked by two ester functions, the ratio between the two respective areas will be smaller than 3. From the ratio of these areas (R), the percentage of cross-linking, x, i.e.

Table 2. Characteristics of Dex-BTC and HES-BTC

	r^a (mmol g^{-1})	r'^a (mmol g^{-1})	$\overline{M}_w$ (LALLS)	Theoretical $\overline{M}_w$	x^c (%)	Sample no.
Dex-BTC	0·6	0·35	13 000	12 400	1·25	D_1
	6	1·25	18 900	16 400	1·5	D_2
HES-BTC	4·5	0·6	39 500	33 000	4	S_1
	7	1	106 000	35 800	17	S_2

[a] As in Table 1.
[b] Calculated by taking into account the BTC content of the substituted polymers as indicated in the text (Results).
[c] Percentage of cross-linking determined by ^{13}C-NMR as described in the text (Results). $\overline{M}_w$ (LALLS) = 11 500 and 28 300 for unsubstituted dextran and HES respectively.

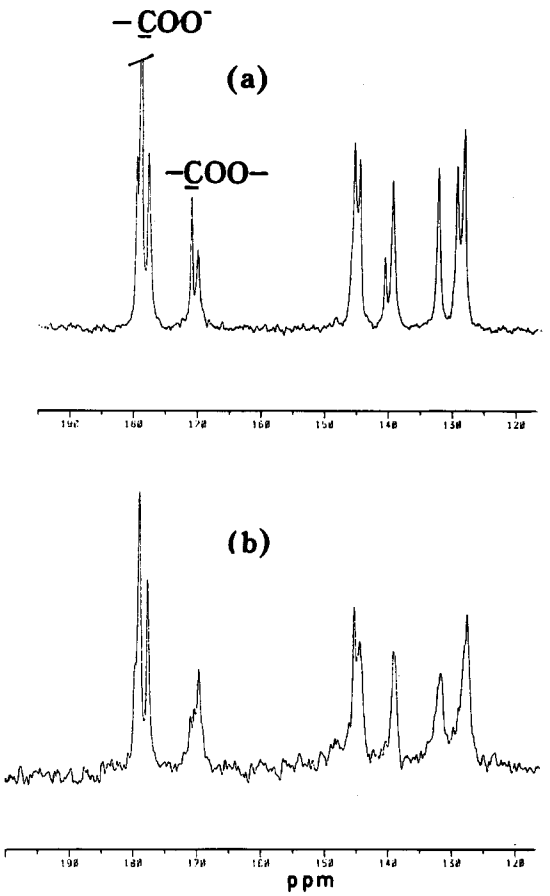

Fig. 2. ^{13}C-NMR spectra of Dex-BTC D_2 (a) and HES-BTC S_2 (b) (see Table 2 for the characteristics of D_2 and S_2).

of BTC groups linked by two ester functions, was calculated by:

$$x = \frac{3 - R}{1 + R} \times 100$$

Figure 2(b) shows the ^{13}C-NMR spectrum of HES-BTC S_2 (Table 2) with the same kinds of peaks around 178 and 171 ppm as for Dex-BTC. Table 2 gives the cross-linking percentages of the HES-BTC samples calculated as described above.

Synthesis and properties of covalent conjugates of Dex-BTC and HES-BTC with oxyHb

The BTC-substituted polysaccharides cleared of free benzene tetracarboxylate were covalently linked to oxyHb following the schematic reaction shown in Fig. 4. The reactivities of Dex-BTC and HES-BTC in this reaction were determined by evaluating the percentages of unmodified Hb remaining at the end of the reactions performed under the same conditions, i.e. 20 mol polymer-linked BTC per mol Hb and 10 mol EDCI per mol Hb. This evaluation was realized from the gel filtration profiles obtained by eluting the reaction media on a TSK G4000 SW column. Figure 5(a) gives the elution profiles of the conjugates obtained from Dex-BTC D_2 and HES-BTC S_2 respectively. The percentages of unmodified Hb, calculated from these chromatograms, were < 2% and 70% respectively. The percentages calculated for the conjugates prepared from D_1 and S_1, from the gel filtration elution profiles shown in Fig. 5(b), were 2% and 17% respectively.

To characterize the oxygen-binding properties of these conjugates, their P_{50} was determined: P_{50} is the partial oxygen pressure for which 50% of Hb is oxygenated. The lower the P_{50}, the higher the Hb affinity for oxygen. At 25°C, pH 7·2, free Hb has a P_{50} of 3·8 torr; in the presence of its natural effector, its P_{50} is about 14 torr. The conjugates obtained from D_1, D_2, S_1 and S_2 (Table 2) were characterized by the P_{50} values listed in Table 3 which were determined on the crude media.

DISCUSSION

From this study, it is clear that the interesting results obtained by modifying Hb with Dex-BTC, i.e. a large increase in the molecular weight of the protein and a significant decrease in its affinity for oxygen (Table 3), cannot be obtained by replacing dextran by HES, another polysaccharidic plasma expander. The strong effect of Dex-BTC on the Hb oxygen affinity was shown to be due to the binding of the BTC-substituted polysaccharide mainly to the amines of the Hb allosteric

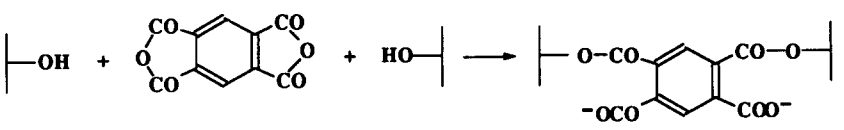

Fig. 3. Mechanism of cross-linking of the polysaccharidic chains by BTCA.

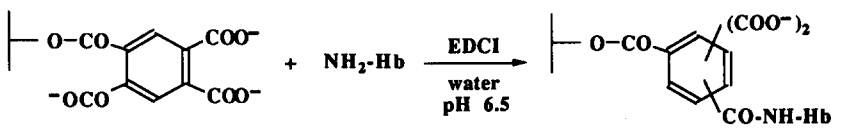

Fig. 4. Schematic reaction of Dex-BTC and HES-BTC with oxyHb.

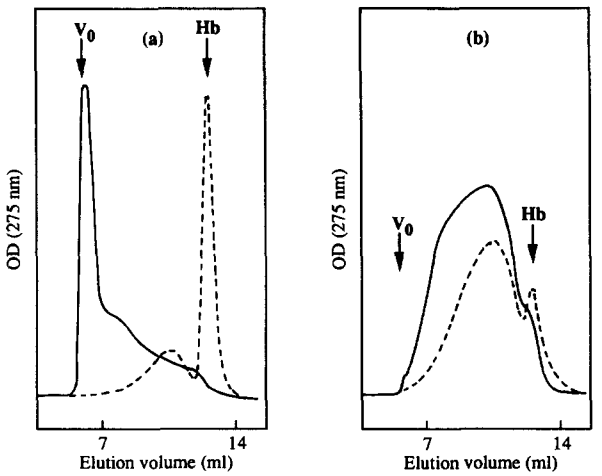

Fig. 5. Gel filtration elution profiles on a TSK G4000 SW column, of the conjugates of oxyHb with various polysaccharides: (a) Dex-BTC D_2 (——) and HES-BTC S_2 (---). (b) Dex-BTC D_1 (——) and HES-BTC S_1 (---). Experimental conditions described in Materials and Methods. The conjugates were prepared with 20 mol polymer-linked BTC per mol Hb and 10 mol EDCI per mol Hb.

Table 3. Oxygen-binding properties of conjugates of oxyHb with Dex-BTC and HES-BTC

Initial polymer	Dex-BTC		HES-BTC	
	D_1	D_2	S_1	S_2
P_{50} (torr)[a]	13·5	14·7	5·3	3·5

[a]Determined at 25°C, pH 7·2 (0·05 M Tris-HCl buffer). The BTC contents of D_1, D_2, S_1 and S_2 are given in Table 2. Conditions of reaction: polymer-linked BTC/Hb = 20 mol mol^{-1}, EDCI/Hb = 10 mol mol^{-1}.

site, due to a strong and relatively specific interaction between the polymer anionic groups and the protonated amines of the Hb allosteric cavity (Prouchayret, 1990).

Since the P_{50} values of the HES-based conjugates are far lower than those based on dextran for similar polymer BTC contents (Table 3), the anionic carboxylate functions of HES-linked BTC are not accessible to the Hb allosteric site. This could be the result of the cross-linking that was evidenced for the HES derivatives by ^{13}C-NMR and by LALLS (Table 2) which would mask the BTC groups. This also explains the smaller reactivity of HES-BTC towards Hb (compared to that of Dex-BTC which is only slightly cross-linked) (Fig. 5(b)), and this is clearly illustrated by the low rate of modified Hb obtained with the highly cross-linked S_2 sample (Fig. 5(a)).

The greater ability of HES to be cross-linked by BTCA compared with dextran is probably the result of the structure of HES which is initially much more branched than dextran T10 (Thomson, 1978; Wurzburg, 1986; Huber, 1991). Moreover, the hydroxyethylation reaction introduces small polyoxyethylene side-chains (Merkus *et al.*, 1977) which can play the role of spacer arms between two HES chains and thus favour the double reaction of one BTCA molecule (Fig. 3).

To limit the extent of cross-linking the reaction of BTCA with HES was performed in more dilute solutions. Thus, for example, while all the Dex-BTC samples were prepared with a dilution of 1 ml water g^{-1} dextran, a dilution of 3 ml water g^{-1} HES was used for

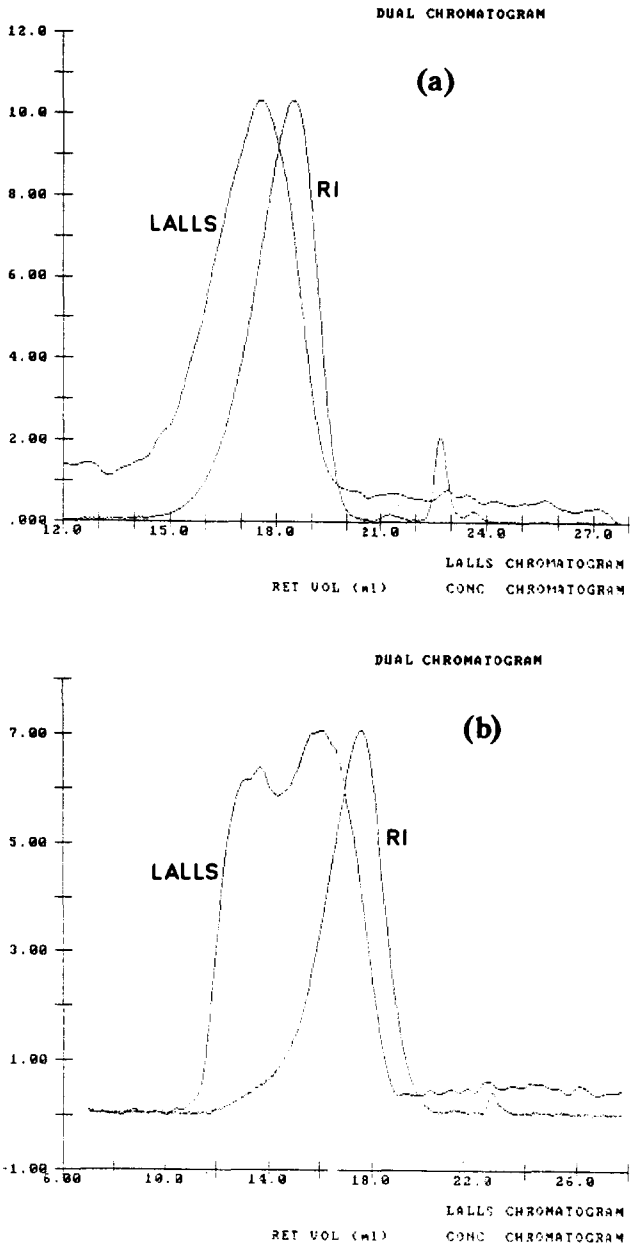

Fig. 6. SEC-LALLS-RI chromatograms of: (a) Dex-BTC (1·25 mmol linked BTC g^{-1} polymer) prepared with a dilution of 1 ml water g^{-1} polymer and (b) HES-BTC (1·2 mmol linked BTC g^{-1} polymer) prepared with a dilution of 40 ml water g^{-1} polymer. Experimental conditions described in Materials and Methods.

HES-BTC S_2 (Table 2) and 18 ml g^{-1} for S_1. Other HES-BTC solutions were prepared with still higher dilutions (up to 40 ml g^{-1}). The cross-linking phenomenon was thus reduced but still occurred, particularly for high contents of BTC, as proved by the SEC-LALLS profile seen in Fig. 6(b) corresponding to a HES-BTC containing 1·2 mmol BTC g^{-1} polymer prepared with 10 mmol BTCA g^{-1} HES with a dilution of 40 ml g^{-1} HES. This profile is to be compared to that of a highly BTC-substituted dextran (sample D_1) for which the LALLS detection does not show any rapidly eluting species with abnormally high molecular weights (Fig. 6(a)).

In conclusion, while HES could be regarded as a better plasma substitute than dextran, with less secondary effects, its BTC derivative is not effective in modifying the oxygen affinity of Hb. In contrast, the BTC derivative of dextran works well in this respect (Dellacherie *et al.*, 1992). Whereas it is possible to link significant amounts of BTC groups to HES, which, in the case of dextran, transforms the polysaccharide into a macromolecular effector of Hb thus capable of decreasing its oxygen affinity (Prouchayret, 1990), the HES resulting derivatives have only a little effect on the protein oxygen affinity because of the existence of highly branched chains which hamper the accessibility of the BTC anionic groups to the protonated amines located in the Hb allosteric site.

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