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Determination of the mean ionic charge of the components of three ^{99m}Tc bone scanning agents by gel chromatography with two eluents of different electrolyte composition

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ABSTRACT

A recently proposed method for the determination of ionic charge was evaluated by applying it to a problem in nuclear medicine, *viz.*, the determination of the mean ionic charge of the components of three ^{99m}Tc bone scanning agents. The method is based on the partition coefficients of the investigated ions, observed in gel chromatography with two eluents containing different electrolytes, at a range of ionic strengths. The bone scanning agents are mixtures of complexes of ^{99m}Tc(III) and ^{99m}Tc(IV) with 1-hydroxyethylene-1,1-diphosphonic acid.

INTRODUCTION

Recently, a method was described [1] for the determination of the charge of ions, based on their partition coefficients observed in gel chromatography with two eluents containing different electrolytes, at a range of ionic strengths. In this paper we report the application of this method to a relevant problem in the field of nuclear medicine, *viz.*, the determination of the (mean) anionic charge of the components of three bone scanning agents.

Briefly, the concept of this method is as follows. As the polarity of the gel is different from that of the eluent, the partition coefficient of any substance is not exactly unity. This is particularly true for (highly charged) ions. As only electroneutral combinations of ions can be transferred from the eluent to the gel and *vice versa*, the partition constant of a sample ion (*i.e.*, of an electroneutral combination with the ions of the electrolyte added to the eluent) depends on the charge z of the sample ion and on the nature of the electrolyte in the eluent. From the combined results in the presence of two different electrolytes, the charge z can be derived. When an ion p^z with charge z is chromatographed in two eluents containing, *e.g.*, $NaClO_4$ and Na_2SO_4, respectively, it holds that

$$\Delta \log K = Cz \tag{1}$$

where $\Delta \log K$ is the difference in the values of $\log K$ in the two eluents (K is the true or thermodynamic value of the partition coefficient) and C is a constant. When C is known and $\Delta \log K$ for an ion with unknown charge z has been obtained, the value of z can be calculated by eqn. 1.

The correction for activity coefficient effects in the experimental result $\Delta \log K'$ was performed as follows. The relationschip between $\Delta \log K$ and $\Delta \log K'$ is

$$\Delta \log K = \Delta \log K' + \log\left[\frac{y_s(NaClO_4)}{y_s(Na_2SO_4)}\right] \tag{2}$$

where $y_s(NaClO_4)$ and $y_s(Na_2SO_4)$ are the activity coefficients of the ion p^z in the $NaClO_4$ and Na_2SO_4

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solutions in the interior of the gel beads, respectively. The extended Debye–Hückel equation yields the following result:

$$\Delta\log K = \Delta\log K' - z^2 A \sqrt{I} f(I,\mathring{a}_i) - f'(c) + C'I \quad (3)$$

where A is a known constant, I is the ionic strength, c is the concentration, $\mathring{a}_i$ is the distance of closest approach of the electrolyte ions to the ion p^z, f and f' are known functional relationships and C' is an unknown constant.

Tji *et al.* [1] measured $\Delta\log K'$ of nine species with z ranging from +2 to −4 on Bio-Gel P-4 at ionic strengths of 0.03, 0.1 and 0.3 M. Making the (not very critical) assumption that $\mathring{a}_i$ is $5 \cdot 10^{-8}$ cm, they calculated the corresponding values of $\Delta\log K' - z^2 A \sqrt{I} f(I,\mathring{a}_i) - f'(c)$. These data were extrapolated to $I = 0$, which yielded the values of $\Delta\log K$. Linear regression of $\Delta\log K$ on z (see eqn. 1) gave the final result:

$$\Delta\log K = (0.106 \pm 0.002)\, z - 0.010 \pm 0.006 \quad (4)$$

This equation can be used as a calibration line for the determination of unknown values of z. In doing so, the value of $\Delta\log K$ should be determined by the same method as used by Tji *et al.* for the determination of the values of $\Delta\log K$ for ions with known charge. A small complication is that the second term on the right-hand side of eqn. 3 depends on z, and that z is unknown. Hence the determination of z must be done by successive approximation.

The new method is based on the assumption that ion pairing between the investigated ion and the ions of the two different electrolytes in the eluents does not occur. This was probably true in the previous investigation [1], and also in the present case, with $NaClO_4$ and Na_2SO_4 as electrolytes and anionic complexes to be investigated.

The theory used in the new method is a bulk-distribution theory. It is not self-evident that it can be applied to gel chromatography, where surface effects may occur. In fact, the results of the previous investigation [1] demonstrated the presence of some carboxylate groups on the gel matrix. To suppress their influence on the distribution of the investigated ions, the ionic strength had to be at least 0.03 M.

A practical point is as follows. In the calculation of K', the total liquid volume in the column must be estimated. The method that we adopted [1] is somewhat arbitrary: the total liquid volume is obtained as the difference between the bed volume and the volume of the gel matrix. The matrix volume is chosen so that $\Delta\log K$ for an uncharged solute (methanol) is equal to zero, as it should be. Other approaches to estimate the total liquid volume in the column are feasible.

Despite these theoretical and practical problems, the precision of the obtained calibration, eqn. 4, gives support for the correctness of the procedure.

The new method had distinct advantages over the classical method for determination of ionic charge, *viz.*, by ion-exchange chromatography. When the investigated ion has a high charge, in the latter method the electrolyte concentration in the eluent must be fairly high to avoid awkwardly long retention times. Corrections for activity coefficients are then virtually impossible, and electrolyte invasion into the ion exchanger cannot be neglected (as is usually done [2]). Further, because of the Donnan equilibrium, the pH in the ion exchanger is higher than that in the eluent, and ions with acidic properties may lose protons when they enter the ion exchanger [3].

The new method also has its limitations. It has been calibrated in the range $-4 \leqslant z \leqslant 2$, and it cannot be excluded that outside this range deviations from the proportionality of $\Delta\log K$ and z occur (in fact, a plot of $\Delta\log K'$ of ribonuclease, obtained by gel chromatography on Sephadex G-75 in eluents containing NaSCN or Na_2SO_4, at ionic strength 0.5 M, *vs.* z is linear in the range $-4 \leqslant z \leqslant 4$, but flattens outside this range [4]). Further, the resolution of mixtures of ions by gel chromatography is low, and it is a fairly slow method. The last point may be a drawback when a mixture of ions (*e.g.*, a mixture of metal complexes) that are interconvertible is investigated.

The performance of the new method in its application to single, stable ions within the calibration range has already been established [1]: unknown values of z can be determined with a standard deviation of 0.18. Here, we give the method a very stringent test by applying it to a relevant problem in the field of nuclear medicine, *viz.*, the determination of the (mean) ionic charge of the components of three ^{99m}Tc bone scanning agents. The agents are mixtures of complexes of $^{99m}Tc(III)$ and $^{99m}Tc(IV)$ with 1-hydroxyethylene-1,1-diphosphonic acid

(HEDP). By high-performance anion-exchange chromatography seven components can be discerned [5]. In gel chromatography only four peaks are observed [6], so it is impossible to determine the charges of the individual complexes by the new method. At most, the mean charge of the complexes in the four gel chromatographic fractions can be determined. The low resolution obtained in gel chromatography may be partly caused by the slowness of the method, as the Tc–HEDP complexes are slowly interconvertible [6].

Even if it turns out that only the mean charge of all the complexes in the bone scanning agents can be determined, the result would be interesting in nuclear medicine. The agents are injected into the blood stream of patients and are taken up by normal bone, and in particular by bone tumours. Any tumours present can be detected by scintigraphy, owing to the γ-radiation that is emitted by ^{99m}Tc. Several workers [7,8] believe that ionic charge is a major factor governing the uptake by the mineral constituent of bone. Hence, it is interesting to investigate whether the mean uptake of the components of the three bone scanning agents is correlated with their mean ionic charge. This might give a clue to further optimization of these agents.

The three ^{99m}Tc–HEDP preparations that were the subject of this investigation have already been shown to have widely different compositions (by high-performance anion-exchange chromatography) and widely different adsorption on $Ca_3(PO_4)_2$ [9].

EXPERIMENTAL

Apparatus and chemicals

Materials were obtained from the following sources: ^{99m}Tc generator (287 mCi Ultratechnekow FM, Mo-99/Tc-99m) from Byk-Mallinckrodt CIL (Petten, Netherlands); HEDP was a generous gift from Henkel (Düsseldorf, Germany); human serum albumin (HSA) from Bloedbank (Utrecht, Netherlands); Bio-Gel P-4 (200–400 mesh) from Bio-Rad Labs., Richmond, CA, USA); chromatography paper (Whatman No. 1) and DC-Alufolien cellulose from E. Merck (Darmstadt, Germany). Chromatographic columns (C10/40), laboratory valves (LV4), reservoir RC-10 and polyethylene capillary tubing were purchased from Pharmacia (Uppsala, Sweden). The Microperpex 2132 pump was obtained from LKB (Bromma, Sweden). The H.V. supply and ratemeter (PW 4620) and the well-type NaI crystal, for on-line detection of radiation, were purchased from Philips (Eindhoven, Netherlands). The two-channel, well-type NaI(Tl) detector counting system was Gamma 8000 system (Beckman Instruments, Irvine, CA, USA).

All other chemicals were of analytical-reagent grade.

Preparation of the reducing agents for the reduction of $^{99m}TcO_4^-$

The following reducing agents were prepared: (1) 0.04 *M* $SnSO_4$ in 0.2 *M* nitrogen-saturated H_2SO_4; (2) 0.04 *M* $FeSO_4$ in 0.2 *M* nitrogen-saturated H_2SO_4; (3) 0.04 *M* $Sn(ClO_4)_2$ in 0.2 *M* nitrogen-saturated $HClO_4$, prepared by mixing 0.08 *M* solutions of $SnSO_4$ and $Ba(ClO_4)_2$ in 0.2 *M* nitrogen-saturated $HClO_4$; and (4) 0.04 *M* $Fe(ClO_4)_2$ in 0.2 *M* nitrogen-saturated $HClO_4$, prepared by mixing 0.08 *M* solutions of $FeSO_4$ and $Ba(ClO_4)_2$ in 0.2 *M* nitrogen-saturated $HClO_4$.

Preparation of bone scanning agents

^{99m}Tc(Sn, pH 7.4)–HEDP and ^{99m}Tc(Sn, pH 12)–HEDP were prepared by mixing 2 ml of 0.4 *M* HEDP (pH 7.4) and 2 ml of reducing agent 1 or 3. The pH was adjusted to 7.4 or 12, respectively, nitrogen was passed through the solution for 5 min and 1.5 ml of the eluate of the ^{99m}Tc generator were added (the generator was eluted with 0.1 *M* Na_2SO_4 or 0.2 *M* $NaClO_4$, respectively). After passing nitrogen through the solution for 15 min, the pH was adjusted to 7.4 and the solution was diluted 400× with Na_2SO_4 or $NaClO_4$ of ionic strength 0.3 *M*.

Samples of this solution were chromatographed with eluents of ionic strength 0.3 *M*. For chromatography with eluents of ionic strength 0.1 and 0.03 *M*, the solutions were diluted 3× and 10×, respectively, with nitrogen-saturated doubly distilled water (the ratio of the concentrations of the HEDP ions in the sample solution and the SO_4^{2-} or ClO_4^- ions in the eluents should be constant at the three different ionic strengths of the eluents. In that case, this ratio will also stay constant, and very small, on extrapolation to zero ionic strength. This is a necessary prerequisite for the application of the calibration function [4]).

^{99m}Tc(Fe)–HEDP was prepared in the same way, but with the use of reducing agents 2 or 4 instead of 1 or 3.

The percentage of radioactivity, present as Tc-HEDP complexes, was determined by Zimmer and Pavel's method [10], slightly modified as described by Kroesbergen *et al.* [11].

Gel chromatography

The eluents of ionic strength 0.3 *M* had the following composition: 0.1 *M* Na_2SO_4 or 0.3 *M* $NaClO_4$, 10^{-3} *M* HEDP, $5 \cdot 10^{-4}$ *M* $SnSO_4$ or $Sn(ClO_4)_2$ (pH 7.4). The eluents of ionic strength 0.1 and 0.03 *M* were prepared by diluting the above eluent 3 × and 10 ×, respectively, with nitrogen-saturated doubly distilled water. All eluents were degassed and filtered before use and kept under nitrogen during chromatography.

The pretreatment of the Bio-Gel P-4 and the packing of the column were performed as recommended by the manufacturer (dimensions 35.5 × 1 cm I.D.).

Aliquots of the samples were applied to the column and eluted at a flow-rate of 10 ml h^{-1}. The flow-rates were accurately determined by weighing the column effluent. The radioactivity was detected on-line by passing the column effluent through a laboratory-made flow cell contained in the well of an NaI deector. The recovery of the applied radioactivity was determined by comparing the radioactivity of a chromatographed sample with that of a non-chromatographed sample.

The volume of the mobile phase was determined by measuring the elution volume of labelled HSA. The labelling was performed as follows. To 3.5 ml of 10% HSA and 0.5 ml of TcO_4^-, 100 μl of $4 \cdot 10^{-3}$ *M* $SnCl_2$ were added and the pH was adjusted to 3.0. The mixture was allowed to react for 10 min. After the pH had been adjusted to 7.4, an aliquot of the mixture was applied to the column. The total liquid volume of the column was obtained from $V_l = V_{bed} - W/\rho$, where V_{bed} is the bed volume, W is the weight of the gel used in the packing of the column and ρ is its density. For ρ an arbitrary value of 1.1 g^{-1} was taken; this value results in a mean value $\Delta\log K_{CH_3OH} = 0$ [1].

Elution volumes were corrected for the extra-column dead space. Partition coefficients were calculated as follows:

$$K' = \frac{V_e - V_0}{V_l - V_0} \tag{5}$$

where V_e is the corrected retention volume, V_0 is the corrected retention volume of HSA and V_l is the total liquid volume in the column. Values of $\Delta\log K'$ were determined at $I = 0.30$, 0.10 and 0.03 *M*. After (partial) correction for the activity coefficients they were extrapolated to zero ionic strength. The charge z was determined from eqn. 4 by successive approximation.

RESULTS

The radioactivity in the complexed fraction was always more than 90%. With Tc(Sn, pH 7.4)–HEDP it was even more than 97%. The main side product was TcO_2; the TcO_4^- concentration was never more than 0.03%. The recovery of radioactivity from the chromatographic column was about 95%.

Representative chromatograms of the three preparations obtained using eluents containing Na_2SO_4 and $NaClO_4$ are shown in Figs. 1 and 2, respectively.

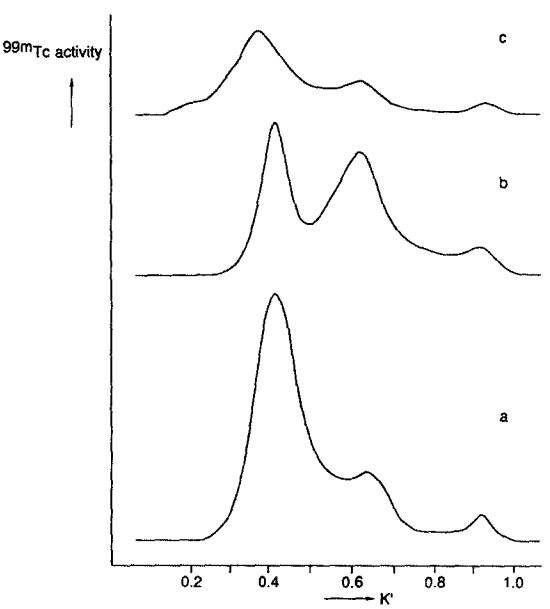

Fig. 1. Chromatograms of (a) ^{99m}Tc(Sn, pH 7.4)–HEDP, (b) ^{99m}Tc(Sn, pH 12)–HEDP and (c) ^{99m}Tc(Fe)–HEDP on Bio-Gel P-4 with an eluent containing Na_2SO_4 of ionic strength 0.3 *M*.

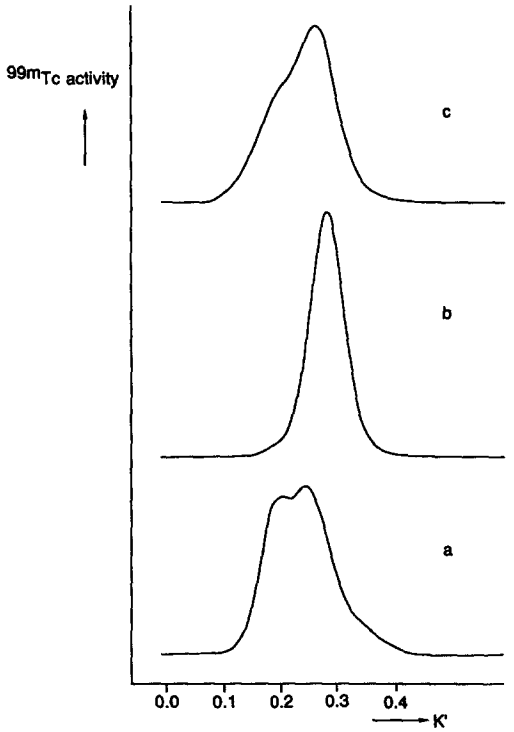

Fig. 2. Chromatograms of (a) ^{99m}Tc(Sn, pH 7.4)–HEDP, (b) ^{99m}Tc(Sn, pH 12)–HEDP and (c) ^{99m}Tc(Fe)–HEDP on Bio-Gel P-4 with an eluent containing $NaClO_4$ of ionic strength 0.3 *M*.

With the eluents containing Na_2SO_4, three fractions can be discerned in all preparations. With the eluents containing $NaClO_4$, Tc(Sn, pH 12)–HEDP and Tc(Fe)–HEDP show only one broad peak. In the chromatograms of Tc(Sn, pH 7.4)–HEDP the early eluting main peak can be discerned. Hence we are able to determine the mean ionic charge of all the components of the three preparations, and in addition the mean ionic charge of the complexes present in the early eluting main peak of Tc(Sn, pH 7.4)–HEDP. The corresponding experimental values of the partition coefficients K' are given in Table I.

Values of the correction terms $z^2A\sqrt{I}f(I,\mathring{a}_i = 5$ Å), for $z = 1, 2, 3$ or 4 (the calculation for other values of z is straightforward), and $f'(c)$ were given by Tji *et al.* [1]. From the values of $\Delta\log K'$ and the above-mentioned correction terms, z was calculated by successive approximation, as described in the Introduction. After 4–6 calculation cycles the differences between the input and output values of z had become very small, ranging from 0.00 to 0.03. The final estimates of z, obtained after 4–6 calculation cycles, are given in Table II.

DISCUSSION

It follows from Fig. 1 that the proportion of the first peak in the chromatograms decreases in the order ^{99m}Tc(Sn, pH 7.4)–HEDP, ^{99m}Tc(Fe)–HEDP, ^{99m}Tc(Sn, pH 12)–HEDP. As a result in Fig. 2 the first peak can only be observed clearly in the chromatogram of ^{99m}Tc(Sn, pH 7.4)–HEDP, and as a shoulder in the chromatogram of ^{99m}Tc(Fe)–HEDP. Probably the poor resolution with the $NaClO_4$-containing eluent is partly caused by the low values of the partition coefficients of the anionic Tc complexes in this eluent. This phenomenon is caused by the high affinity of the gel for ClO_4^-. For the first peak in the chromatogram of ^{99m}Tc(Sn, pH 7.4)–HEDP $K' = 0.20$ and 0.42 when the eluent contains $NaClO_4$ and Na_2SO_4 of ionic strength 0.3

TABLE I

PARTITION COEFFICIENTS, K', OF THE EARLY-ELUTING MAIN PEAK OF ^{99m}Tc(Sn, pH 7.4)–HEDP, AND MEAN VALUES OF K' OF THE THREE BONE SCANNING AGENTS (OBTAINED BY DIVIDING THE CHROMATOGRAMS IN TWO PARTS OF EQUAL AREA), IN ELUENTS CONTAINING $NaClO_4$ OR Na_2SO_4 OF IONIC STRENGTH 0.03, 0.1 AND 0.3 *M*

Species		$NaClO_4$			Na_2SO_4		
		0.03 *M*	0.1 *M*	0.3 *M*	0.03 *M*	0.1 *M*	0.3 *M*
^{99m}Tc(Sn, pH 7.4)–HEDP	Main peak	0.20	0.17	0.20	0.49	0.42	0.42
	Mean	0.22	0.19	0.23	0.54	0.45	0.45
^{99m}Tc(Sn, pH 12)–HEDP	Mean	0.26	0.24	0.28	0.63	0.57	0.57
^{99m}Tc(Fe)–HEDP	Mean	0.26	0.23	0.24	0.53	0.43	0.42

TABLE II

FINAL VALUES OF ΔLOG K AND z, OBTAINED BY SUCCESSIVE APPROXIMATION, AND THEIR STANDARD DEVIATIONS

The standard deviations of $\Delta\log K$ follow from regression analyses according to eqn. 3. The standard deviations of z are obtained from error propagation using the standard deviations of $\Delta\log K$ along with regression parameters of eqn. 4.

Species		I (M)	$\Delta\log K'$	Corr.[a]	$\Delta\log K$ (I = 0)	z
^{99m}Tc(Sn, pH 7.4)–HEDP	Main peak	0.03	−0.39	0.16	−0.51 ± 0.004	−4.8 ± 0.1
		0.1	−0.39	0.22		
		0.3	−0.47	0.32		
	Mean	0.03	−0.38	0.21	−0.60 ± 0.03	−5.6 ± 0.3
		0.1	−0.38	0.29		
		0.3	−0.29	0.43		
^{99m}Tc(Sn, pH 12)–HEDP	Mean	0.03	−0.38	0.20	−0.58 ± 0.02	−5.4 ± 0.2
		0.1	−0.37	0.27		
		0.3	−0.31	0.40		
^{99m}Tc(Fe)–HEDP	Mean	0.03	−0.31	0.09	−0.39 ± 0.01	−3.6 ± 0.1
		0.1	−0.27	0.13		
		0.3	−0.25	0.19		

[a] Corr. = $z^2 A \sqrt{If}(I, \mathring{a}_i = 5\ \text{Å}) + f'(c)$

M, respectively. The equation for chromatographic resolution contains a factor $k'/(1 + k')$, where k' is the capacity factor, which is equal to the product of K' and the ratio of the volumes of stationary and mobile phase in the column. In the present instance, this ratio is 1.74, so for K' = 0.20 or 0.42, $k'/(1 + k')$ = 0.25 or 0.42. The ratio of these values is 0.60. As the resolution is also proportional to the square root of the column length, the drawback of low K' values with the $NaClO_4$-containing eluent might have been offset by increasing the column length by a factor of 3. However, in the present case of slowly interconvertible complexes, this would probably cause additional problems.

The standard deviation of the results for z is small, but it must be noted that they are slightly out of the calibration range of the method [except the result for ^{99m}Tc(Fe)–HEDP].

A comparison with literature data obtained by ion-exchange chromatography is only possible for the mean charge of all the components of ^{99m}Tc(Sn, pH 7.4)–HEDP. Huigen *et al.* [12] used two ion exchangers, Aminex A-28 and DEAE-Trisacryl, and applied two methods, described by Wilson and Pinkerton [13] and by Russell and Bischoff [3] (the latter of which is probably more accurate). By Wilson and Pinkerton's method they obtained z = −8 and −4, respectively, and by Russell and Bischoff's method they obtained z = −7 and −6, respectively. Our, probably more accurate, value is z = −5.6.

The correlation between the values of z for the three bone scanning agents and their adsorption on the mineral constituent of bone is discussed elsewhere [9].

CONCLUSION

A recently proposed method for the determination of ionic charge has been demonstrated to be useful in a complicated case in the field of nuclear medicine: the determination of the mean ionic charge of the components of three ^{99m}Tc bone scanning agents [these agents are mixtures of at least seven complexes of ^{99m}Tc(III) and ^{99m}Tc(IV) with 1-hydroxyethylene-1,1-diphosphonic acid, that are slowly interconvertible]. For ^{99m}Tc(Fe)–HEDP the mean ionic charge is −3.6 ± 0.1 at pH 7.4; for ^{99m}Tc(Sn)–HEDP, prepared at two widely different pH values, 7.4 and 12, the mean ionic charges at pH 7.4 are −5.6 ± 0.3 and −5.4 ± 0.2, respectively. For the complexes eluting in the first peak of the gel chromatogram of ^{99m}Tc(Sn, pH 7.4)–HEDP the mean ionic charge is −4.8 ± 0.1.

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