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Calibration of gel-permeation columns in the high-molecular-mass range: Fixed human thrombocytes for the estimation of interstitial volume and the haemocyanin of the Vineyard snail *Helix pomatia* as a molecular mass calibration substance

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Abstract

Human thrombocytes, fixed with formaldehyde, are shown to be a universal indicator of the interstitial volume in gel-permeation chromatography (size-exclusion chromatography) for gels of all molecular mass separation ranges. The fixed thrombocytes are simple to prepare and to handle.

Furthermore, native or intramolecularly cross-linked haemocyanin of the Roman snail *Helix pomatia* is shown to be well suited as a molecular mass calibration substance. We propose $8.8 \cdot 10^6$ as its molecular mass for calibration. This haemocyanin can therefore be added to the list of globular protein molecular mass calibration substances in the range of very high molecular masses.

1. Introduction

The aim of our research is the development of a new kind of an artificial oxygen transporter based on polymerized haemoglobins. Preliminary measurements with gel-permeation chromatography (GPC) have shown soluble hyperpolymeric haemoglobins with broad distributions of molecular masses which are partially greater than $6 \cdot 10^6$ [1–4]. Two problems arise in the measurement of values and distributions of those molecular masses with GPC: firstly, suitable high-molecular-mass calibration substances are needed, and secondly, for a gel-specific calibration, valid for all lengths of columns and qualities of packing, a simple assessment of the interstitial volume (V_0) is necessary. Suitable gels are available, e.g. Sephacryl S-500 HR, Sephacryl S-1000 SF and Sepharose CL-2B from Pharmacia (Freiburg, Germany).

One phenomenon of GPC, when different substances of the same class are used, is that there is a linear relationship between a distribution coefficient, e.g. the volume fraction (FV), and the logarithm of the molecular mass (log M) of the substance. FV is calculated as $(V_e - V_0)/(V_t - V_0)$, where V_e is the elution volume and V_t the total mobile phase volume. The latter is easily assessed by using small molecules, in this case vitamin B_{12}. The ratio V_t/V_T of vitamin B_{12}

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is 0.97 (with Sepharyl S-500 HR), where V_T is the total (geometrical) volume of the column.

However, a reliable, practical and sufficiently accurate measurement of V_0 of a column, filled with the gels as mentioned above, is a problem. A substance is needed whose molecules are, at least partially, greater than the greatest pores of the separation gel, thus allowing the determination of the absolute exclusion limit of the gel. Synthetic polymers with broad molecular mass distribution, for example Blue Dextran 2000 with a mean molecular mass of $2 \cdot 10^6$, are often used. Applying this substance to gels with a separation range for medium molecular masses results in a definite exclusion maximum, but with gels having separation ranges of high molecular masses, Blue Dextran 2000 is not suitable for indication of the interstitial volume.

Manufacturers of these gels do not offer suitable substances for measurement of the interstitial volume, but instead recommend killed bacteria or viruses, which are not readily available to all investigators. Calculation of the interstitial volume as a fraction of the total column volume, as also recommended for GPC in the high-molecular-mass separation range, is insufficient for an accurate determination of molecular masses under the actual conditions of chromatography, e.g. quality of filling of the column, type of eluent, flow-rate, temperature, dimensions of the column, kind of specimen, etc.

Therefore, we here describe a method for determination of the interstitial volume using fixed human thrombocytes, which can readily be prepared in every biomedical laboratory at any time.

Ideal molecular mass calibration substances should be of uniform molecular mass and similar in shape and molecular density as compared to the substances to be analyzed. For our purpose we use native proteins, which should be of very high molecular mass. Uniform "giant" proteins do exist, for example the erythrocruorin of the earth-worm *Lumbricus terrestris*. This erythrocruorin is suitable as a molecular mass calibration substance [5], but its molecular mass is "only" $3.34 \cdot 10^6$. Therefore, the use of the haemocyanin of the snail *Helix pomatia* (Roman snail) as a molecular mass calibration substance in GPC is also described. Its molecular mass is much higher, with light-scattering and sedimentation measurements yielding $7.55 \pm 0.5 \cdot 10^6$ [6] and $9 \cdot 10^6$ [7], respectively.

2. Materials and methods

2.1. Production of fixed human thrombocytes

Venous blood is taken from a cubital vein using a plastic syringe, previously flushed with a heparin solution (5000 IU/ml). The heparinized blood is centrifuged in 10-ml polycarbonate tubes for 5 min with a relative centrifugal acceleration of 500 *g*. The supernatant is the so-called platelet-rich plasma (PRP). In a polyethylene reaction vessel, 150 μl of 37% formaldehyde solution are added to 1 ml of PRP at room temperature and mixed, the mixture is then allowed to react for 24 h. To prevent formation of gels, the suspension is diluted to one third after this time, preferentially with the elution medium used: the protein concentration is then approximately 2 g/dl.

2.2. Preparation of haemocyanin

The shell of the snail is opened by filing a hole on the right side of the shell at the beginning of the second winding — above the marginal lung vein. The aperture is widened by carefully breaking off the edges of the hole with a pair of tweezers. The visible vein is then opened with a pointed pair of scissors or a scalpel, the opalescent haemolymph flows out and can be collected into a small tube. Pressing back the body of the snail into its shell with a finger through the natural aperture enhances the yield considerably: up to 2 ml of the haemolymph, containing 3 g/dl haemocyanin [8], may be obtained. Alternatively, although with lower yield, the haemolymph may be aspirated from the marginal vein using a cannula. Normally, the snails survive this procedure. Before use for calibration, the haemolymph is diluted to one third, preferably

with the eluent, and filtered through a 0.45-μm filter.

2.3. Intramolecular cross-linking of haemocyanin

A 3-μmol amount of divinyl sulfone (Sigma, Deisenhofen, Germany; 30 μl of a freshly prepared 0.1 mol/l solution in electrolyte BIKU, see below) are added to 1 ml of the diluted haemocyanin solution (ca. 1% in concentration) or to 300 μl of the original haemolymph. After 24 h at room temperature, 60 μmol lysine (60 μl of a 1 mol/l solution in electrolyte BIKU) are added to the mixture and the whole solution is allowed to react for another 24 h.

2.4. Chromatography

The columns used were 1 cm in diameter and about 80 cm in length. The flow-rate of the aqueous eluent was 5.25 ml/h and detection was carried out in flow-through cuvettes with a dead volume of about 50 μl and an optical pathlength of 1 cm. Haemoglobin and erythrocruorin were detected at a wavelength of 425 nm, all other proteins and calibration substances at 275 nm. Chromatographic gels were Sepharose CL-2B, Sephacryl S-400 HR, Sephacryl S-500 HR and Sephacryl S-1000 SF, all purchased from Pharmacia (Freiburg, Germany). Eluents were isosmotic (with plasma) aqueous electrolytes compounded as follows: (1) BIKU: 125 m*M* NaCl–4.5 m*M* KCl–20 m*M* $NaHCO_3$, pH 8.5; (2) HENA: 144 m*M* NaCl–10 m*M* 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES buffer), pH 7.4; and (3) Helix-Ringer: 63 m*M* NaCl–4.2 m*M* KCl–13.2 m*M* $MgCl_2$–10.3 m*M* $CaCl_2$–10 m*M* Tris buffer, pH 7.5. All three contained 0.2 g/dl NaN_3.

Volume fractions (FV) were calculated as mentioned above. The interstitial volume (V_0) was determined with Blue Dextran 2000 (Pharmacia) or with fixed human thrombocytes, and the total mobile phase volume (V_t) with vitamin B_{12} (Sigma, Deisenhofen, Germany). Calibration proteins and their molecular masses were: ribonuclease A 13 700, haemoglobin 64 500, bovine serum albumin 67 000, alcohol dehydrogenase 141 000, ferritine 440 000, thyroglobulin 669 000 (all from Pharmacia), and *Lumbricus terrestris*-erythrocruorine $3.34 \cdot 10^6$.

3. Results

3.1. Measurements of V_0

Fig. 1A and B show original chromatograms of determinations of the interstitial volume using the same column, filled with Sephacryl S-400 HR. Determinations were carried out with Blue Dextran 2000 (Fig. 1A) and with fixed PRP (fPRP) as the indicator (Fig. 1B) for comparison. The interstitial volumes, determined from these chromatographic runs, were 31.04 and 30.59 ml, respectively.

Fig. 1C and D show analogous original chromatograms using Sephacryl S-500 HR gel. It is evident that Blue Dextran 2000 is not sufficient to indicate the interstitial volume (Fig. 1C), whereas the suspension of fixed thrombocytes is (Fig. 1D). Finally, Fig. 1E and F show analogous results on a column filled with Sephacryl S-1000 SF; the interstitial volume is clearly indicated by the fixed thrombocytes (Fig. 1F), but not by Blue Dextran 2000 (Fig. 1E).

The values of the interstitial volume, determined with both indicators on Sephacryl S-400 HR, did not differ significantly. Corresponding values of the other gels are in the same range. The standard errors are small and, hence, relative standard deviations are also small, lying between 1 and 3%. Table 1 summarizes the results for all gels used.

3.2. Haemocyanin as calibration substance

The haemocyanin of the snail *Helix pomatia* was eluted on all gels used without any sign of dissociation into subunits, always with Helix-Ringer as eluent, and for comparison also with HENA on Sephacryl S-400 HR. Fig. 2 shows a typical original chromatogram with Sephacryl S-400 HR. With all gels the FV–log *M* functions were measurable, using the mentioned calibra-

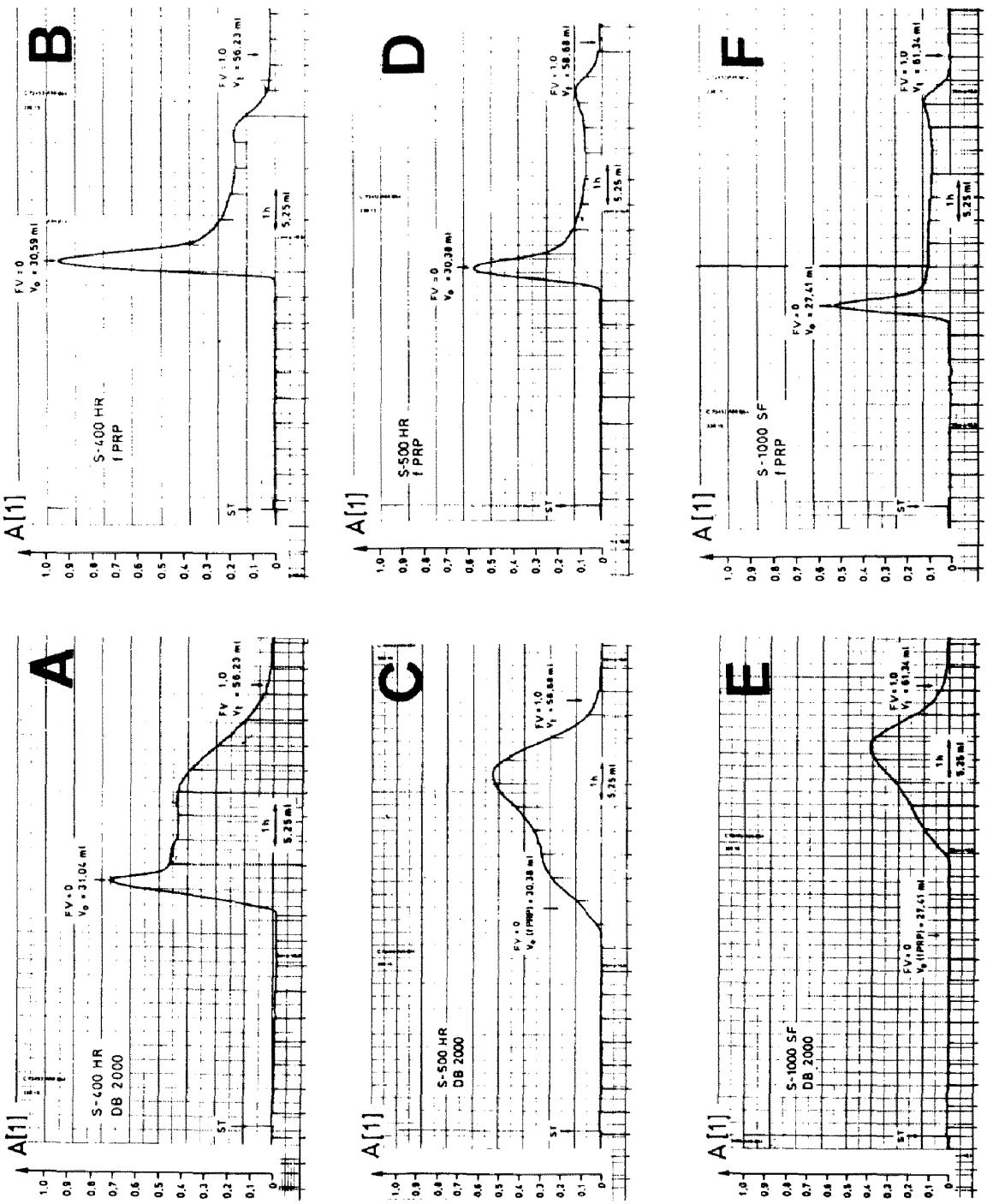

Fig. 1. Examples of original gel-permeation chromatograms of Blue Dextran 2000 (DB 2000: A, C and E) and fixed human thrombocytes (fPRP: B, D and F) for the indication of V_0 on Sephacryl gels S-400 HR (A and B), S-500 HR (C and D) and S-1000 SF (E and F). Eluent HENA; ST = start; A = absorbance.

Table 1
Values for interstitial volume (V_0) of columns packed with different gels, measured with Blue Dextran 2000 (DB 2000) and fixed human thrombocytes (fPRP)

GPC-gel	S-400 HR		S-500 HR	S-1000 SF
V_0 indicator	DB 2000	fPRP	fPRP	fPRP
V_0 (ml)	31.044	30.586	30.019	28.587
	30.815	30.356	29.894	27.476
	30.806	29.255	30.837	28.014
	30.753	29.308	30.380	27.956
	29.156	30.296		27.081
				28.330
$\bar{V}_0$ (ml)	30.52	29.96	30.28	27.91
$s_{\bar{V}_0}$ (ml)	0.77	0.63	0.42	0.56
$s_{\bar{V}_0}/\bar{V}_0$	0.03	0.02	0.01	0.02

$\bar{V}_0$ = Arithmetic average; s_{V_0} = standard deviation; $s_{\bar{V}_0}/\bar{V}_0$ = relative standard deviation.

tion proteins together with haemocyanin; Fig. 3 shows the results. Within the error of measurement, the functions are approximately linear, and all corresponding correlation coefficients are greater than 0.992. The molecular mass of haemocyanin ($8.3 \cdot 10^6$) used for these calculations, was taken as the mean from light-scattering measurements ($7.55 \cdot 10^6$ [6]) and from sedimentation measurements ($9 \cdot 10^6$ [7]).

When using BIKU (pH 8.5) as the eluent on Sephacryl S-400 HR, the chromatograms of the haemocyanin are analogous to those of mixtures of proteins of lower hydrodynamic volumes. The mean elution volumes correspond to a molecular mass of about $1.5 \cdot 10^6$. This may be explained by the well known dissociation of the haemocyanin into subunits in solutions with pH values different from the physiological one. Such subunits are still complexes of the smallest subunits [9]. In contrast, haemocyanin treated with divinyl sulfone has the same elution volume in all eluents used.

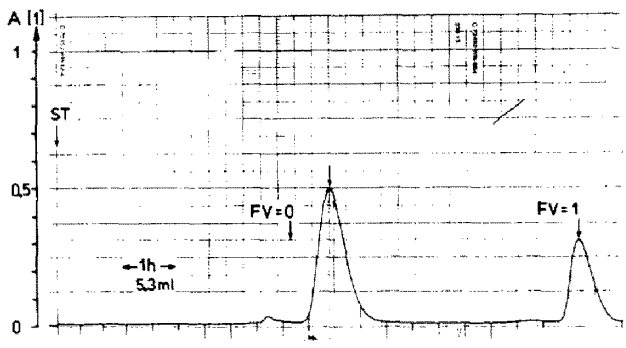

Fig. 2. Gel-permeation chromatogram of haemocyanin from the snail *Helix pomatia* on Sephacryl S-400 HR gel. Eluent Helix-Ringer; ST = start; A = absorbance.

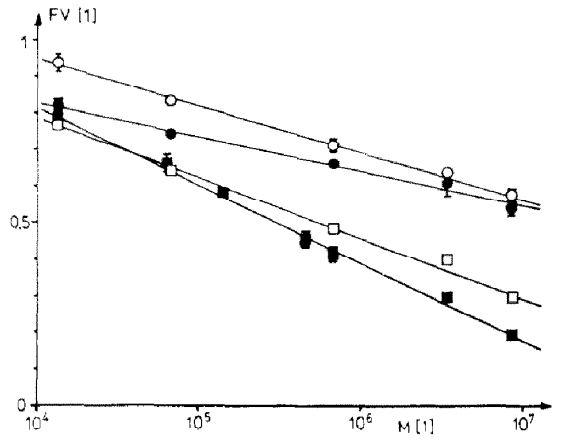

Fig. 3. FV–log M calibration curves of native proteins on different GPC gels, and their coefficients of correlation r. ○ = Sepharose CL-2B, $r = -0.998$; ● = Sephacryl S-1000 SF, $r = -0.993$; □ = Sephacryl S-500 HR, $r = -0.997$; ■ = Sephacryl S-400 HR, $r = -0.999$. Eluent was HENA for Sephacryl S-400 HR, for all other gel types it was BIKU for *Lumbricus*-erythrocruorin, Helix-Ringer for *Helix*-haemocyanin, and HENA for the other proteins. The lengths of the bars indicate the standard deviations ($n = 3$–7).

4. Discussion

The results presented here show that fixed human thrombocytes are suitable for determination of the interstitial volume. Dilution of the thrombocytes after fixation is recommended to avoid gelatinization, which could result in column obstruction. In the event of gelatinization, occurring even at this recommended dilution, repeated preparation with greater dilution is advised. Normally a suspension of fixed thrombocytes is stable for months. But following prolonged storage, the suspension should be prefiltered, for example through a 0.8-μm filter, in order to remove invisible minute gel particles, which may have formed.

The Sephacryl gels investigated here are similar in their chemical structure and gel particle size. They differ only in porosity, and thus in their range of separation of molecular volumes. Therefore it can be assumed that interstitial volumes, determined with fixed thrombocytes on Sephacryl S-500 HR and on Sephacryl S-1000 SF are accurate (accuracy of the mean) since with Sephacryl S-400 HR the same values are obtained (within the limits of measurement error), as determined with Blue Dextran 2000 and with fixed thrombocytes.

A determination of the interstitial volume with fixed thrombocytes is also possible on gels with different chemical structure, for example on Sepharose CL-2B. Five measurements of V_0 resulted in a mean of 26.03 ml and a standard deviation of 0.18 ml. On this gel, Blue Dextran 2000 is again not sufficient for the indication of V_0.

Fixed human thrombocytes are probably also suitable as V_0 indicators for other gel types, and fixed thrombocytes from animals may also be suitable for this purpose.

With one column filling many determinations of the interstitial volume and analytical runs are possible alternately, always with reproducible values. This shows that there are no specific interactions between fixed thrombocytes and the gels, and, especially, gel columns do not become obstructed.

Haemocyanin from the snail *Helix pomatia* is eluted in Helix-Ringer and in HENA with no sign of dissociation; chromatograms resemble those of molecular homogeneous substances. Under these conditions there seems to be no dissociation into subunits, which has been described for different degrees related to ionic strength, acidity and concentration of Ca^{2+} [9]. Molecular masses determined under the conditions described here are always maximal values, and therefore probably correspond to the undissociated molecule.

To our knowledge, Largier and Polson [10] are the only investigators who have used a complete haemocyanin as a calibration substance in GPC with an aqueous eluent. It was the haemocyanin of a maritime snail, *Burnupena cincta*, with a molecular mass of $6.6 \cdot 10^6$. This snail is only found in the sea around South Africa and thus its haemocyanin is not easy to obtain. In addition, the molecular mass of this haemocyanin is relatively low.

Calibration functions determined using the haemocyanin of *Helix pomatia* are almost linear, with corresponding correlation coefficients close to one. However, as Fig. 3 also shows, in all gels except Sepharose gel, the volume fractions for the earth-worms erythrocruorin lie above the calibration line; those for the haemocyanin lie below it. Because GPC separates molecules according to their hydrodynamic volumes, it follows, that the molecular volume of the erythrocruorin is "too small" and that of the haemocyanin is "too large". A possible explanation is that the molecular density of the erythrocruorin is higher than that of the haemocyanin. In accordance with this, the molecular mass of the haemocyanin, as assessed by measured volume fractions and the mean (linear) calibration line of the other calibration points, is higher than that measured by sedimentation and with light scattering. With the Sephacryl gels S-400 HR, S-500 HR and S-1000 SF, and with Sepharose CL-2B we calculated the molecular masses to be $9.8 \cdot 10^6$, $10 \cdot 10^6$, $12 \cdot 10^6$ and $8.3 \cdot 10^6$, respectively. The mean molecular mass of haemocyanin regarding all three methods (sedimentation, light scattering and GPC) is $8.8 \cdot 10^6$. Despite the discussed systematic deviations, which

are within the limit of the measurement error, the linearity of the calibration curves legitimizes the use of the haemocyanin of *Helix pomatia*, which is easily accessible and easy to handle, as a calibration protein in GPC. It can therefore be added to the list of calibration proteins in the high-molecular-mass range.

Nevertheless it would be useful to have more values of the molecular mass of this haemocyanin, assessed by light scattering and/or with sedimentation, especially in the ionic medium used in this investigation. We propose to take the above-mentioned value $8.8 \cdot 10^6$ as the molecular mass for calibration, which is the average of all available values.

*Intra*molecular cross-linking of the haemocyanin using divinyl sulfone as described above does not produce detectable *inter*molecular cross-links, in contrast to analogous treatment (addition in the same molar ratio to haemocyanin) with glutaraldehyde, another bifunctional cross-linker for proteins. Its reaction products contain polymers of the haemocyanin or its subunits, which makes the reaction products unsuitable for molecular mass calibrations. The relative amount of divinyl sulfone, as mentioned above, is the minimum necessary for a complete intramolecular stabilization, as evidenced by chromatography.

Some other proteins, catalase, ferritin and thyroglobulin, when treated in an analogous manner with divinyl sulfone (application in the same mass ratio related to the proteins), are also not polymerized (results not shown here).

As an extension of the application of the erythrocruorin from the earth-worm *Lumbricus terrestris* as a calibration substance in GPC [5], erythrocruorins from the near-related earth-worms *Lumbricus rubellus* and *Dendrobena veneta* may be used alternatively. These erythrocruorins have the same molecular mass and all of these earth-worms are available from shops specializing in fishing equipment.

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