STUDY OF MODIFIED BOVINE SERUM BY GEL FILTRATION ON PEARL-CONDENSED AGAR

T. I. PŘISTOUPIL AND S. ULRYCH
Institute of Hematology and Blood Transfusion, Prague (Czechoslovakia)*
(Received February 25th, 1966)

In a previous communication[1] we referred to gel filtration on Sephadex G-200 of an experimental blood volume expander[2–4] prepared by heat denaturation and formolation of bovine serum in the presence of partially degraded gelatin and by the oxidation of this product with hydrogen peroxide. The above mentioned method of fractionation showed the modified bovine serum (MBS) to consist mainly of three groups of denatured and chemically modified protein molecules with different molecular sizes[1]. The main peak of the elution curve, however, was already near to the exclusion limit of the Sephadex G-200. For further investigation of this high molecular fraction of MBS by gel filtration it seemed inevitable that another gel with a looser structure would have to be used. Granulated and pearl-condensed agar, which has been used with success by several authors in recent years[5–10], seemed to be suitable for our purpose. In the present paper we report and discuss some results obtained during our experiments with gel filtration of MBS on agar pearls under different conditions.

MATERIALS AND METHODS

MBS was a standard preparation, batch No "DG 472", prepared in our laboratory[1–3]. Proteins used for calibration (bovine ribonuclease, human serum albumin, human γ-globulin, human thyreoglobulin) were the same preparations as had been used previously[1]. Tobacco mosaic virus (TMV) was a gift of Ing. M. ČECH from the Institute of Experimental Botany, Czechoslovak Academy of Science. A standard buffer 0.1 *M* tris-(hydroxymethyl)-aminomethane (TRIS) + 1 *M* NaCl, pH 8.0 (adjusted by HCl), was used in these experiments.

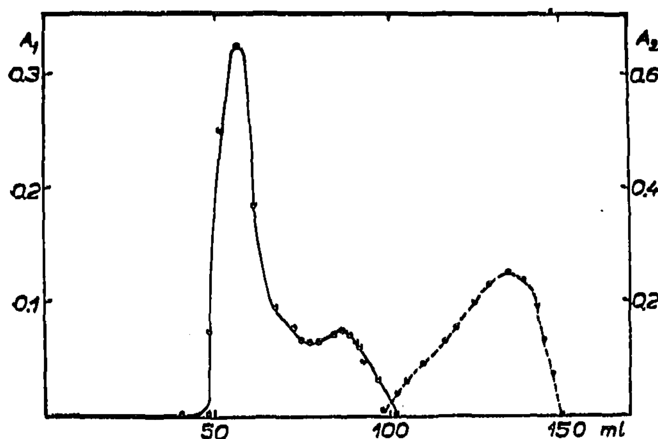
Agar pearls were prepared as follows (*cf.* ref. 8): A hot solution (about 90°) of 4 % Difco agar in water (200 ml) was poured at once into a cool (20–25°) mixture of toluene (316 ml), tetrachloromethane (86 ml) and Tween 60 (0.2 ml) and the mixture was subjected to vigorous mechanical stirring for about 30 min. After cooling to laboratory temperature and washing with water, the suspension of gel particles was sieved, agar pearls of 25–100 mesh were equilibrated with the buffer and used to fill the column. The column was 1.4 cm in diameter and 84 cm high. A 1.6 × 78 cm column of Sephadex G-200 was used for comparative experiments. The samples were applied on the top of the column in volumes of 2 ml. The protein concentration was 1.0–4.5 %. The flow rate was 2.5–5 ml/cm²/h at 20–25°. Fractions of 2–4 ml were

* Director: Prof. J. HOŘEJŠÍ, M.D., D.Sc.

collected using a simple automatic collector[11] and were analysed for proteins either by direct photometry at 280 nm on a Zeiss VSU Universal spectrophotometer or turbidimetrically after a reaction with trichloroacetic acid or tannin (estimation of oxygelatin) as mentioned before[1]. The void volume was determined by using diluted Indian ink[10]. Viscosity measurements of MBS samples with a protein concentration of 1.8 % were made on an Ubbelohde viscometer at 20°.

RESULTS AND DISCUSSION

The differences between the elution patterns of MBS on Sephadex G-200 and on 4 % pearl-condensed agar, respectively, are obvious when comparing Figs. 1 and 2. On Sephadex G-200 (Fig. 1) three peaks could be detected (*cf.* ref. 1); the front part of the first main peak, containing particles of molecular weights up to 10^6 according to sedimentation analysis[12], was very steep and without any mark of fractionation since it was close to the void volume. On agar, however, fractionation of the MBS into at least four groups of macromolecules was observed (Fig. 2). The elution volume of the first small fraction was close to the void volume thus indicating the presence of particles with a relatively large size corresponding presumably to mol. wt. of the order of 10^6. As the presence of a large amount of such particles could cause some decrease in the therapeutic value of MBS, we wanted to get more information on this fraction. It was of special interest to examine whether the fraction A in Fig. 2 consisted of stable macromolecules (like condensation products of proteins and formaldehyde[4]) or merely aggregates stabilized transitorily by the actual composition of the



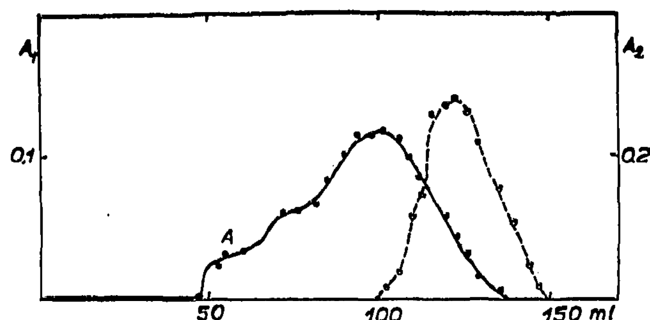


Fig. 1. Gel filtration of MBS on Sephadex G-200. Column size 78 × 1.6 cm; buffer 1 *M* NaCl + 0.1 *M* TRIS, pH 8.0. A_1 = absorbance at 280 nm; A_2 = absorbance from the turbidimetric estimation of oxygelatin[1] (dotted line).

Fig. 2. Gel filtration of MBS on pearl-condensed 4 % agar. Column size 84 × 1.4 cm. For other data see Fig. 1.

buffer used under the given conditions. To examine the influence of salt concentration upon the aggregation of protein molecules of MBS, the buffer was diluted with water 1:50 and 1:100 and gel filtration of MBS, dialysed against the diluted buffers, was performed on agar pearls which had also been equilibrated with those buffers.

According to the results shown in Fig. 3, the first peak A increased as a result

of the increased dilution of the buffer. This could be interpreted as an aggregation of the modified proteins at a low salt concentration, the aggregation being due most probably to electrostatic forces (salt bridges). This interpretation seemed to be con-

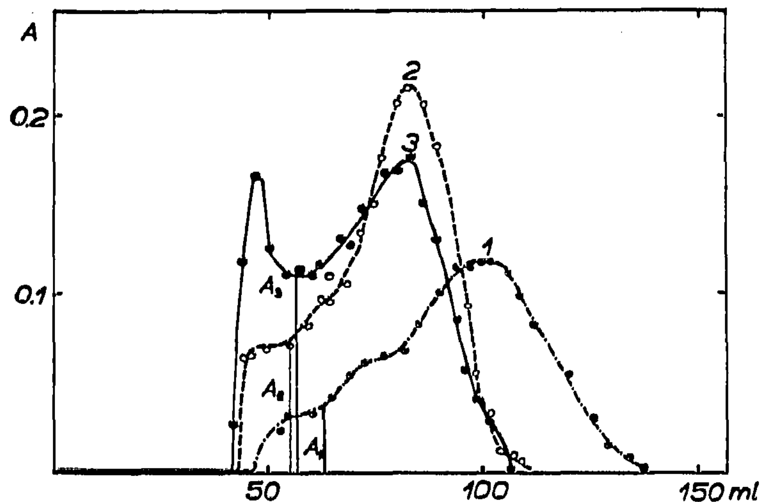


Fig. 3. Gel filtration of MBS on agar pearls at different salt concentrations. (1) Buffer 1 *M* NaCl + 0.1 *M* TRIS, pH 8.0; (2) the standard buffer (1) diluted 1:50; (3) the standard buffer (1) diluted 1:100.

firmed further by viscosity measurements of MBS. As demonstrated in Fig. 4 the relative viscosity of MBS showed a strong dependence on the buffer concentration when extreme dilutions were approached. These salt-concentration dependent changes of viscosity were found to be reversible. At physiological salt concentration (0.9 % NaCl), the viscosity of MBS was practically the same as in the undiluted buffer and also the corresponding elution curve was very similar to curve 1 in Fig. 3. This led to the conclusion that under physiological conditions the first fraction of MBS contained relatively stable macromolecules.

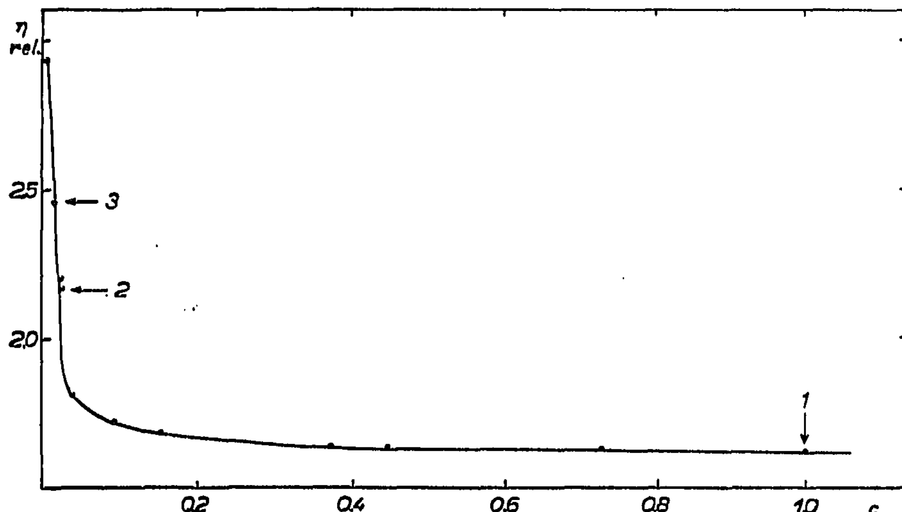


Fig. 4. The dependence of viscosity of MBS on salt concentration. Dots indicated by the arrows correspond to curves 1, 2 and 3 in Fig. 3.

The calibration curve (Fig. 5) constructed by plotting the elution volumes of the standard proteins against their molecular weights on a semilogarithmic scale was used for a rough estimate of the molecular weights of some of the MBS fractions in the range up to about $7 \cdot 10^5$. Thus, for oxygelatin (G) and the main peak of MBS (M), molecular weights of about 15,000 and 300,000, respectively, were found. The same caution over interpretation, however, had to be taken as was referred to earlier[1] when MBS, *i.e.* denatured molecules and particles of different shapes and sizes[5,13], was analysed. With regard to this, it seemed reasonable to expect that the molecular weights of G and M would in fact be substantially less than indicated above (*cf.* ref. 1).

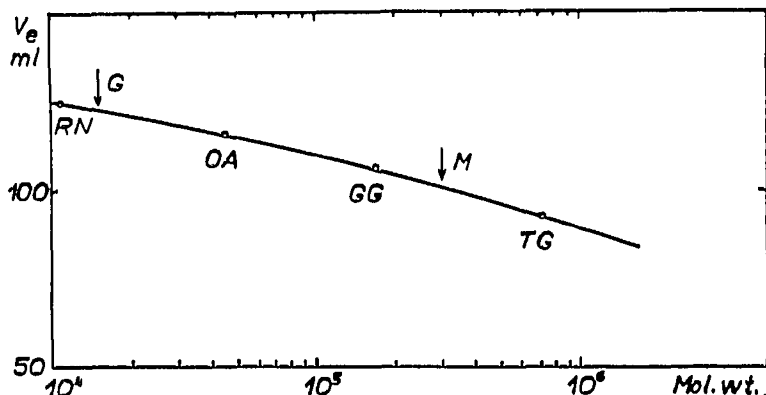


Fig. 5. Calibration curve of molecular weights of proteins on pearl-condensed 4% agar. RN = bovine ribonuclease; OA = ovalbumin; GG = human γ-globulin; TG = human thyreoglobulin. V_e of gelatin (G) and of the main peak of MBS (M) are indicated by arrows.

With respect to the remaining two peaks of MBS it could only be stated that their molecular weights were of the order of 10^6. Since there were no appropriate standards at our disposal to extend the calibration curve further and the V_e of TMV was practically at the V_v of the column, the above statement was based on comparing the following V_e data (*cf.* Fig. 3 and Fig. 5): Indian ink (42 ml), TMV (43 ml), first peaks of curves 2 and 3 in Fig. 3 (47 ml), first peak of MBS (55 ml), second peak of MBS (74 ml), thyreoglobulin (92 ml).

To get a quantitative (although relative) measure for characterising the content of the high molecular fraction in a given batch of MBS, the elution curves were evaluated as follows (see Fig. 3): The areas A_1, A_2 and A_3 (limited by the ordinate, the corresponding part of the curve and the abscissa intersecting the given curve at the "minimum" between the first and the second peak) were expressed as percentages of the total area limited by each elution curve and the ordinate. The following results were obtained after evaluating the curves on Fig. 3: 8 % for curve 1, 11 % for curve 2 and 29 % for curve 3. This method of evaluation offers a new method of checking the standard of quality of MBS and of studying the correlation between the content of the high molecular weight fraction and the biological effect of different batches of MBS.

ACKNOWLEDGEMENT

The authors are grateful to Miss J. NOVOTNÁ for technical assistance.

SUMMARY

Denatured and chemically modified serum proteins of an experimental blood volume expander were characterised by gel filtration on pearl-condensed 4 % agar gel. The presence of at least 4 protein fractions was demonstrated. The salt-concentration dependence of the aggregation of the modified proteins was studied by gel filtration and viscometry. A simple way of quantitatively evaluating the elution curves was proposed as a possible means for checking the standard of the quality of MBS.

REFERENCES

1 T. I. PŘISTOUPIL, *J. Chromatog.*, 19 (1965) 64.
2 T. I. PŘISTOUPIL, J. NIKL, V. FRIČOVÁ AND A. HRUBÁ, *Probl. Gematol. i Pereliv. Krovi*, (1966) 32.
3 T. I. PŘISTOUPIL, V. FRIČOVÁ AND E. PALUSKA, *Z. Immunitaets- Allergieforsch.*, 125 (1963) 50.
4 T. I. PŘISTOUPIL, *Experientia*, 21 (1965) 194.
5 A. POLSON, *Biochim. Biophys. Acta*, 50 (1961) 565.
6 J. KILLANDER, S. BENGTSSON AND L. PHILIPSON, *Proc. Soc. Exptl. Biol. Med.*, 115 (1963) 861.
7 S. BENGTSSON AND L. PHILIPSON, *Biochim. Biophys. Acta*, 79 (1964) 399.
8 S. HJERTÉN, *Biochim. Biophys. Acta*, 79 (1964) 393.
9 J. F. LARGIER AND A. POLSON, *Biochim. Biophys. Acta*, 79 (1964) 629.
10 M. ČECH, *Virology*, 18 (1962) 487.
11 T. I. PŘISTOUPIL AND V. FRIČOVÁ, *Chem. Listy*, 58 (1964) 667.
12 Z. HRKAL, unpublished results.
13 P. ANDREWS, *Biochem. J.*, 96 (1965) 595.