With the substituted maltosides the order of migration varied, *viz.* deoxy-iodo > deoxy > tosyl > acetate and the sequence was found to be general; for example, compound 5 > 6 > 4 > 2. This sequence occurred also with 6-substituted methyl α- and β-glucopyranosides[3]. EVANS *et al.*[4] report for 6-chloro-6-deoxy and 6-deoxy methyl D-glucopyranosides a sequence deoxy > chlorodeoxy > hydroxy. With both the acetylated and unacetylated chlorodeoxy and deoxy glucosides in several solvent systems we observed the sequence chlorodeoxy > deoxy > acetate (or hydroxyl). No explanation can be offered for this difference.

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Thin-layer gel filtration of modified proteins on 4% fine pearl-condensed agar

The possibility of preparing fine pearl agar suitable for thin-layer gel filtration has been reported in some preliminary results[1]. The major advantages of this thin-layer technique are reduced running time and very small sample size; the latter is especially important if we are working with high molecular weight substances which have been prepared by complicated and difficult isolation methods.

The fractionation experiments to test the feasibility of thin-layer gel filtration on agar gel were performed with nonprotein material–Blue Dextran 2000.

In the present study we tried to use the same thin-layer method for simple orientation estimation of aggregate and molecular sizes of modified and denatured proteins of an experimental blood expander "Modified Bovine Serum" (MBS)[2–4]. This protein material is a heterogeneous and complex mixture of modified serum protein and gelatin, together with their aggregates and degradation products.

Gel filtration on agar, resp. agarose gel, which has been introduced by POLSON[5], was shown to be more suitable for fractionation of MBS than gel filtration on Sephadex G-200. Dextran gel has too rigid a structure and its network detains only a smaller part of the comparatively large molecules and aggregates of MBS.

Materials and methods

"Modified Bovine Serum" was a standard preparation, batch No. "DG 472", prepared by heat denaturation and formolation of bovine serum in the presence of

partially degraded gelatin and by oxidation of this product with hydrogen peroxide in our laboratory. Proteins and Blue Dextran 2000 used for calibration were commercial preparations, bovine ribonuclease (Reanal, Hungaria), human serum albumin and human gamma globulin (Institute of Sera and Vaccines, Prague), Blue Dextran 2000 (Pharmacia, Uppsala). α_2-Macroglobulin was a gift from Mr. ŠTEFEK of the Research Institute for Immunology, Prague.

A standard buffer, 0.1 *M* Tris + 0.1 *M* NaCl adjusted to pH 7 by HCl, was used in the experiments for both gel filtration on columns and thin-layer techniques. The preparation of fine agar pearls by a modified method according to HJERTÉN[6] has been described previously[1]. The pearls having a 40–100 mesh size were used for gel filtration on the column and those of 100–400 mesh for the thin-layer technique.

Gel filtration was performed on a column of 4% agar pearls, equilibrated with the solution of standard Tris buffer. The column was 1.4 cm in diameter and 90 cm high. A 2 ml portion of the 4% MBS was applied to the top of the column and the flow rate was adjusted to 10 ml/h. Fractions of 4 ml were collected and measured after a reaction with trichloroacetic acid. The fractions of MBS selected for thin-layer gel filtration were concentrated by dialysis against 20% dextran solution.

The thin-layers of agar pearls were prepared on 8 × 15 cm glass plates, thickness of the layers being 0.5 mm. The samples (20 μl of 0.5–2% protein solution) were applied with a micropipette as round spots 5 mm in diameter. The flow rate through the gel of 1.5 cm/h was regulated by the angle of the plate (approx. 20°) to horizontal. After completion of a run the substances were transferred from the gel layer by adsorption into a sheet of a slightly wet filter paper. After 1 min the sheet was removed, dried for 10 min and stained for 30 min by 0.1% Nigrosine. The migration distances of the spots of proteins were measured from their start to their centre and expressed as D_r values[7] (ratio of the migration distance of the retarded species to that of the excluded species).

Results and discussion

Fig. 1 shows a typical elution curve of MBS without degraded gelatin on 4%

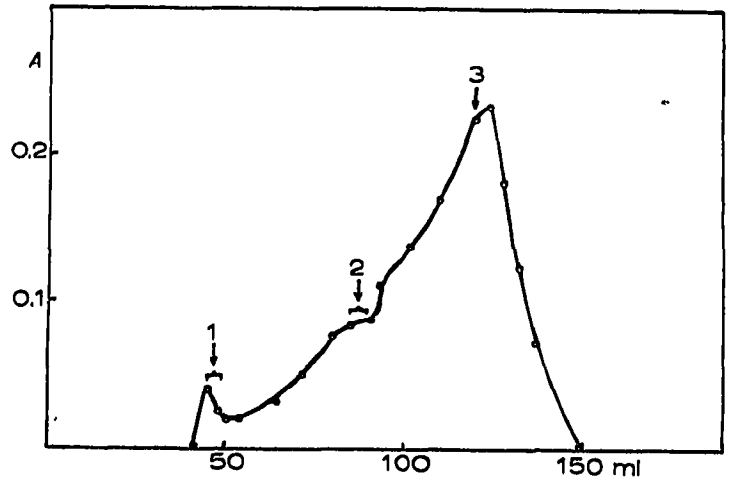

Fig. 1 Column gel filtration of MBS on pearl-condensed agar, particle size 40–100 mesh. Column 90 × 1.4 cm, buffer 0.1 *M* Tris + 0.1 *M* NaCl + HCl, pH 7. The fractions selected for thin-layer gel filtration are indicated by arrows

pearl-condensed agar[8]. On the agar column, only a small part of the MBS is in the region of the exclusion limit, which is different from gel filtration on Sephadex G-200 (ref. 9), where MBS is almost totally excluded. The main part of the MBS is considerably slowed down on agar. Thus the structure of 4% agar gel is more convenient for the fractionation of the large molecules of the modified proteins of bovine serum than the structure of Sephadex G-200. The elution volume of the first small peak, which consists of relatively large particles corresponding to a mol. wt. of the order of 10^6, is close to the void volume of the agar column. The main peak according to the calibration curve was found to have a mol. wt. of about 300,000.

Since modified proteins of MBS possess such a wide range of sizes they seemed to be suitable for testing thin-layer gel filtration on 4% pearl agar, when we take into consideration that for example the commercial product Sepharose 4 B (Pharmacia, Uppsala)[10] with approx. 4% agarose has a fractionation range of mol. wt. of about 300,000–3×10^6 (booklet 1967) and the exclusion limit for protein mol. wt. is about 20×10^6 (booklet 1969).

Thin-layer gel filtration of MBS and its column fractions on 4% agar gel can be seen on Fig. 2. Only the first fraction from column gel filtration had an almost equal length of run to Indian ink, which was used for the determination of the exclusion limit of the plate. The other fractions of MBS and unfractionated MBS were distinctly slowed down compared with Indian ink. A short table of D_r values of MBS and its fractions on 4% agar gel and Sephadex G-200 is given below (Table I).

If we compare the D_r values in Table I we can see that thin-layer gel filtration

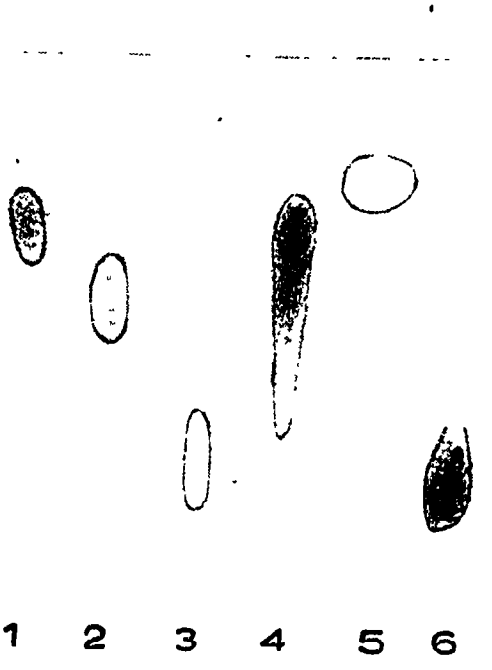

Fig. 2. Thin-layer gel filtration on agar of the MBS fractions from column gel filtration. Pearl-condensed agar, 4%, particle size 100–400 mesh. Plates 8 × 14 cm, thickness of the layers 0.4 mm, buffer 0.1 *M* Tris + 0.1 *M* NaCl + HCl, pH 7, flow rate 1.5 cm/h, 20 µl of 0.5–2% protein solution applied. 1, 2, 3 = fractions from Fig. 1, 4 = unfractionated MBS, 5 = Bromphenol Blue, 6 = dilution of Indian ink.

TABLE I

D_r VALUES OF MBS AND ITS FRACTIONS

Sample	*4% agar*	*Sephadex G-200*
MBS	0 62	1 00
Fraction 1	0 98	1.00
Fraction 2	0 58	1.00
Fraction 3	0 42	0.80

on agar pearl has advantages over the same technique on a dextran gel of the type Sephadex G-200 for fractionation of the large size molecules of MBS. All the fractions of MBS are separated on agar gel which may be used to separate molecules and particles up to a molecular weight of several millions. Only the third fraction is different from the exclusion limit and can be identified on Sephadex G-200.

We were interested in the possibility of a rough estimation of molecular (particle) sizes directly from the data of the thin-layer technique. For this purpose we calibrated the agar gel plate with various proteins of known molecular weight and calculated the D_r value for each protein (Fig. 3). A linear relationship is seen to exist when the D_r values are plotted against the log of the molecular weight of the retarded protein, a relationship similar to that described by ANDREWS[11]. The calibration curve was considerably steeper than that for the same proteins on Sephadex G-200. D_r values of standard proteins and Blue Dextran 2000 on 4% agar pearl and Sephadex G-200 are shown in Table II.

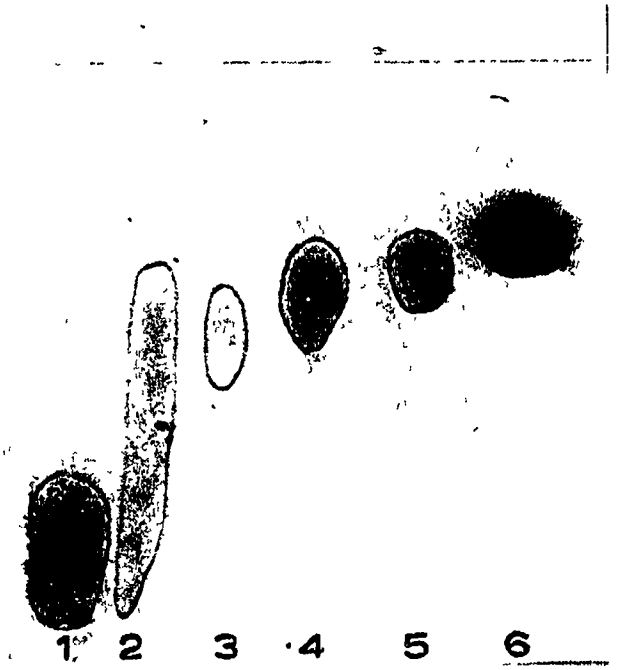

Fig. 3. Thin-layer gel filtration on agar of: 1, Indian ink (the second spot is probably the protein of the glue that is in commercial Indian ink); 2, Blue Dextran 2000. 3, α_2-macroglobulin, 4, human γ-globulin; 5, human serum albumin; 6, bovine ribonuclease. Experimental conditions see Fig. 2.

TABLE II

D_r VALUES OF STANDARD SUBSTANCES

	Mol. wt	D_r *values*	
		4% agar	*Sephadex G-200*
Ribonuclease	13,000	0.36	0.35
Serum albumin	69,000	0.43	0.60
γ-Globulin	156,000	0.48	0.81
α_2-Macroglobulin	820,000	0.57	1.00
Blue Dextran 2000	2,000,000	0.76	1.00

The first fraction of MBS according to the calibration curve was found to have a molecular weight of the order 10^6, the second fraction 700,000–800,000 and the third fraction 50,000–60,000.

Our results presented in this communication show that under suitable conditions thin-layer gel filtration on pearl-condensed agar can serve as a simple and convenient method for the orientation estimation of relatively large molecular sizes of modified proteins.

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