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CHARACTERIZATION OF NATIVE MULTICOMPONENT PROTEIN MIXTURES BY ONE- AND TWO-DIMENSIONAL GRADIENT ELECTROPHORESIS

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SUMMARY

An arrangement of one- and two-dimensional polyacrylamide gradients has been applied to the analysis of multicomponent mixtures of soluble proteins: (1) two-dimensional 3–25% slabs; (2) 3–40% cylinders; and (3) 20–50% cylinders. The run in the first dimension is performed in non-restrictive agarose gel to ascertain a "pure" charge abscissa. Gradient electrophoresis is performed over prolonged periods in order to reach or closely approach the exclusion (pore) limit, which is taken as an operational parameter for the hydrodynamic size of globular molecules irrespective of their frictional ratios. For each of the three gradients one to four reference mixtures were formulated that allow size evaluations of non-denatured proteins. Human serum again proved its usefulness as a universal reference mixture for newly developed techniques. The number of stainable components is in accordance with expectation based on the broad knowledge of serum proteins. Two-dimensional agarose/gradient electrophoresis seems to fulfil the one band–one protein strategy of high-resolution techniques.

INTRODUCTION

The retardation of protein–SDS (sodium dodecyl sulphate) complexes in polyacrylamide gel electrophoresis depends primarily on the molecular mass, because charge differences are diminished by the acidity of the detergent and the molecular weights of single constituents from complex protein mixtures can be easily determined[1–6]. Under the conditions usually applied, however, composite proteins that are kept together by non-covalent bonds dissociate into their subunits. As many biological fluids contain substantial amounts of high-molecular-weight proteins, their subunits then intermingle inseparably with the native low-molecular-weight proteins. An analogous technique that combines non-dissociating running conditions with the sieve effect of gel electrophoresis may therefore represent an attractive analytical tool.

After long running times on gel gradients, many proteins approach zero mobility[7,8] and the final position depends primarily on the hydrodynamic size[9].

Slowly migrating proteins, however, may never reach their exclusion (pore) limits within reasonable running times, thus leading to an overestimation of their molecular size. This source of error, which is unavoidable in one-dimensional techniques, can be overcome by a preceding free-zone electrophoresis, which sorts out all proteins that migrate below a limiting velocity.

The running position in agarose accurately reflects the free electrophoretic mobility, as the effective pore radius of a 1% gel is several times greater than the largest serum protein β-lipoprotein ($R = 12.4$ nm) and it is justified to consider the first dimension as the charge axis of a two-dimensional analytical grid. The non-restrictive agarose gel has many advantages. It can be brought into any convenient shape, allows two-directional separations, adheres tightly to the polyacrylamide gel surface and the difference of electroendosmosis in both gels can be overcome by appropriate running conditions.

Isoelectric focusing of multicomponent mixtures in synthetic ampholytes reveals microheterogeneities of many individual proteins that often cannot be clearly differentiated from true compositional heterogeneities. On application of human serum, which is the best known biological protein mixture, we were unable to localize precisely the position of several well known individual proteins, as any particular spot may represent either an individual protein or a charge isomer without functional significance.

The agarose/pore gradient map reveals a smaller number of spots, but each individual protein appears as a characteristic entity that can be confined by specific staining, immune techniques and analytical reasoning, thus taking advantage of the charge and size coordinates. The technique would therefore fulfil the "one spot–one protein" condition that remains the ultimate goal of high-resolution methods.

EXPERIMENTAL AND RESULTS

Separation according to size was performed on linear gel gradient slabs with total monomer concentrations from 3 to 25% ($T = 3$–25%). These withstand the long running times necessary to reach (or at least approach) zero mobility. Small-pore 5-mm cylinder gels with monomer concentrations up to 50% have been used for small molecular proteins with molecular weights above 3000 daltons. The total pore size range (3–50%) was then divided and for technical reasons the following compositions were chosen for standard experiments in glass tubes: large pore gradient, $T = 3$–40%, $C = 5$–10%; small pore gradient, $T = 20$–50%, $C = 10\%$ (T = total monomer concentration; C = portion of crosslinking monomer).

Micro-scale gradients of comparable low porosities have already been produced, utilizing density differences, capillary forces and lateral diffusion[10].

As in column chromatography the analytical validity of the method depends strongly on the calibration system utilized[11]. Each constituent of a universal reference mixture should fulfil the following requirements:

(1) overall globular conformation with frictional and axial ratios that are representative of the respective molecular size group[9];

(2) sufficient mobility to approach the exclusion limit with reasonable running times;

(3) high solubility in the running buffer to provide narrow starting zones;

(4) no interactions with other components of the mixture;
(5) commercial availability for a reasonable price in a highly purified state.

A limited polymerization tendency increases the number of values for the reference curve. After extensive studies, the proteins compiled in Table I and under *Calibration mixtures* were selected for calibration purposes. The finally adopted two-dimensional reference mixture contained lactoglobulin, albumin, transferrin, catalase and ferritin (Fig. 1). The following proteins of human serum are easy to recognize and can be used as additional references: prealbumin, α_2-macroglobulin and β-lipoprotein (Fig. 2). A representative calibration graph is shown in Fig. 3, and it is evident that the oligomers of lactoglobulin, albumin, transferrin and ferritin share the overall correspondence between hydrodynamic volume and molecular weight that is characteristic of "normal" globular proteins.

TABLE I

MOLECULAR DATA OF SELECTED REFERENCE PROTEINS

See also under *Calibration mixtures*. The values were compiled from refs. 12–16 and the literature sources cited in a previous paper[9]. MW = molecular weight; R = hydrodynamic radius; $\bar{R}$ = mass equivalent geometric radius for $\bar{v} = 0.73$ g/ml; pI = isoelectric point; R_F = relative mobility as referred to bromophenol red in 0.2 M barbital buffer (pH 8.6); $R/\bar{R}$ = frictional ratio; $R/\bar{R}_r$ = frictional ratio representative of "normal" globular proteins of the respective size. Note the tendency to more space-occupying conformations with molecular size.

Protein	*MW*	*R (nm)*	*pI*	R_F	$R/\bar{R}$	$R/\bar{R}_r$
Ribonuclease	13,700	1.64	8.9		1.08	1.14
Whale myoglobin	16,890	1.86	8.2	19	1.11	1.16
Lactoglobulin-dimer	35,830	2.90	5.3		1.33	1.23
Ovalbumin	43,500	2.74	4.7		1.18	1.22
α-Amylase	48,600	2.67		27	1.11	1.24
Prealbumin	61,000	3.25	4.7	81	1.29	1.24
Albumin	69,000	3.58	4.9	67	1.27	1.26
Transferrin	81,000	3.67	5.9	31	1.30	1.27
Fumarase	206,000	5.27	7.7		1.27	1.31
Catalase	241,200	5.22	5.7	45	1.27	1.32
Ferritin	473,450	7.90	4.5	55	1.53	1.35
Thyroglobulin	669,000	8.60	4.5		1.48	1.36
α_2-Macroglobulin	798,000	9.35	5.4	44	1.49	1.36
β-Lipoprotein	2,239,000	12.40		33	1.46	1.42

Technical details of the two-dimensional procedure

First-dimensional electrophoresis of four samples is performed in a 1.5-mm layer of 1% agarose (Litex Co., Glostrup, Denmark) on a 10 × 10 cm slide. The pre-warmed samples, containing 0.3–0.8 mg of protein, are mixed 1:1 with 2% agarose at 52° and 1 μl of bromophenol red solution (saturated solution diluted 1:5). Gel slits of appropriate sizes 2 cm apart from the cathode edge are filled completely with 20–80 μl of the gel–sample mixtures. The running buffer is 0.2 M barbital buffer (pH 8.6).

Filter-paper wicks (Whatman No. 1) are applied as current bridges with contact zones of 1.1 cm at the cathode side and 1.7 cm at the anode side. A potential of 250 V is applied until the dye band has reached the edge of the filter-paper. The

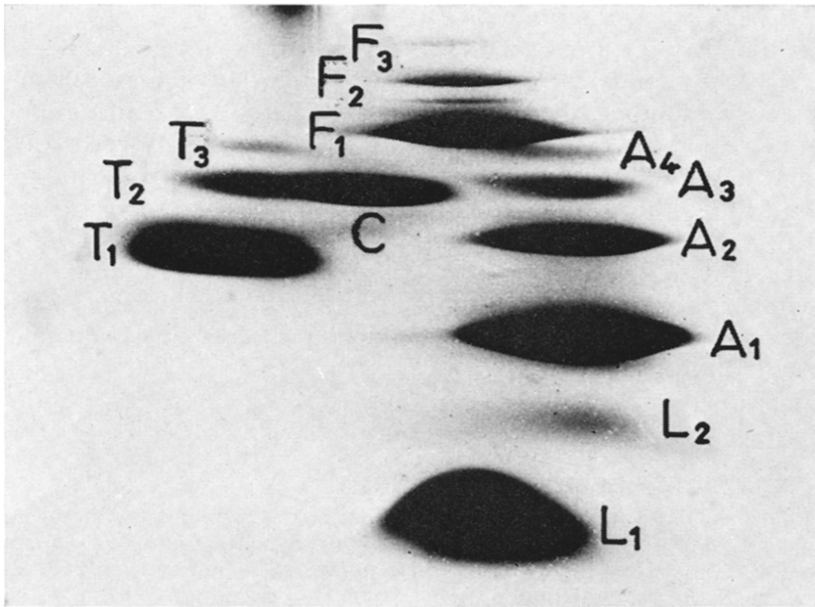


Fig. 1. Reference mixture for the two-dimensional technique. Relative mobilities of the monomers (R_F of bromophenol red = 100): albumin A = 67, lactoglobulin monomer L = 61, ferritin F = 55 (α_1), catalase C = 45 (α_2), transferrin T = 31 (β). The polymers of transferrin migrate increasingly faster than the monomer. Note a still faster migrating macromolecular transferrin aggregate at the large pore edge of the slab.

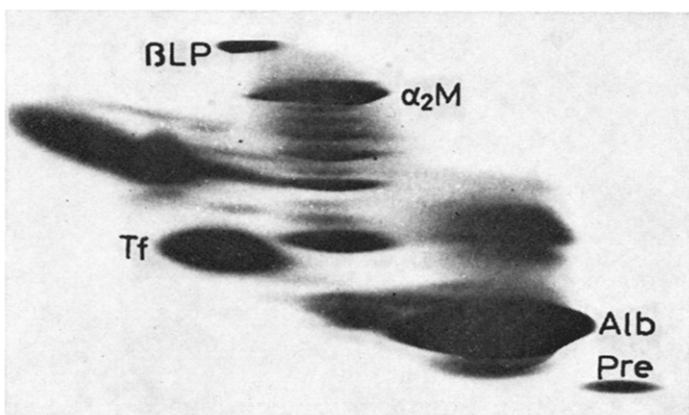


Fig. 2. Proteins of human serum that can be easily recognized for calibration purposes. The R_F values have been determined in agarose at pH 8.6 and the free mobilities m_0 (cm²/V · sec) on barbital buffer pH 8.6 (β-lipoprotein in phosphate buffer pH 7.8) were taken from Schultze and Heremans[17]. Prealbumin, $R_F = 81$ ($m_0 = 7.6$, p$I = 4.7$); albumin, $R_F = 67$ ($m_0 = 5.9$, p$I = 4.9$); α_2-macroglobulin, $R_F = 44$ ($m_0 = 4.2$, p$I = 5.4$); β-lipoprotein, $R_F = 33$ ($m_0 = 3.1$); transferrin, $R_F = 31$ ($m_0 = 3.1$, p$I = 5.9$). Note the size-heterogeneous α_1-lipoprotein family above albumin and the comma-like appearance of the charge heterogeneous γ-immunoglobulins.

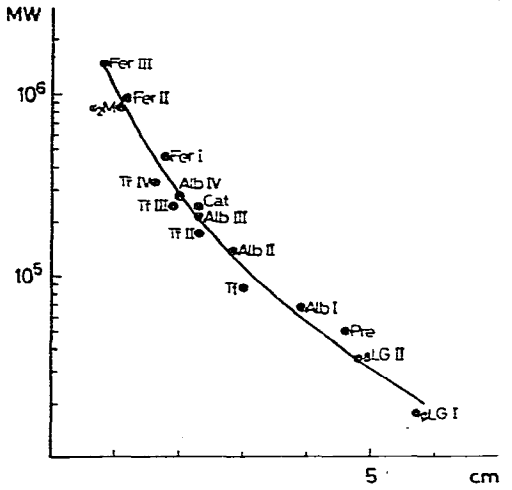


Fig. 3. Calibration graph for a representative two-dimensional slab gradient, $T = 3$–25%, $C = 5\%$. The values were measured on slabs with the standard reference mixture and human serum. βLG = β-lactoglobulin; Pre = prealbumin; Alb = albumin; Tf = transferrin; Cat = catalase; Fer = ferritin; α_2M = α_2-macroglobulin.

gel is cut at this height, thus fixing the mobility reference ($R_F = 100$). Allowance for the endosmotic flow can be made by adding 20 μg of vitamin B_{12} to the sample. Individual strips (1.4 × 7.2 cm) are excised without touching the surface of the gel.

The residual fluid is carefully removed from the surface of the polyacrylamide gel with filter-paper. Flat air cushions between the glass and gel surface can be cautiously expelled by manual pressure from the outside. The agarose strip is slowly pushed into the gradient gel chamber with the help of a slide-rule, leaving a 1-mm gap. All remaining space is quickly filled with 1% agarose without entrapping air bubbles.

For the second-dimensional run a Pharmacia GE-4 chamber apparatus is used. Leaky gaskets are sealed with 1% agarose to prevent a current by-pass. The running buffer is 0.089 *M* Tris–0.082 *M* borate buffer (pH 8.6) containing 0.0025 *M* EDTA.

Electrophoresis is performed for 24 h with a constant current of 40 mA. The buffer, permanently circulated, is renewed after 12 h. The gradient chambers are placed for 5 min in an incubator at 80° to soften the adhesive (see below), immersed in water and opened with the help of a scalpel.

The staining solution is 10 g of Coomassie Brilliant Blue R 250 (Serva, Heidelberg, G.F.R.), 900 ml of water, 900 ml of methanol and 200 ml of glacial acetic acid. The solution is stirred overnight and filtered. The slabs are stained for 24 h and de-stained in 10% acetic acid–50% methanol with slight agitation. The de-staining solution is replaced several times. The gels are stored in air-tight plastic envelopes.

Preparation of the slab gradients

Plastic spacers (2 × 4 × 83 mm) are fixed on thoroughly cleaned 83 × 83 mm glass slides with a thermoplastic adhesive (UHU plus Endfest 300, UHU-Werk,

Bühl, G.F.R.). Eight gradient chambers are placed side-by-side in the Pharmacia GSC 8 casting apparatus and the monomer solution is introduced from the bottom. The single compartment volumes are determined with water, the following amounts being representative for an eight chamber experiment: 35 ml of water (agarose space), 88 ml of 3% acrylamide (half the chamber volume) and 103 ml of 25% acrylamide (half the chamber volume plus the dead volume).

Linear gradients are prepared with the Ismatec MP 13 GI-10 13-channel tubing pump (Ismatec SA, Zürich, Switzerland). The mixing chambers are kept in an ice-bath, the whole arrangement is protected from UV light and room temperature is kept between 20° and 22°. The initial pumping speed is set at 130 ml/h and increased stepwise to 220 ml/h after 15-min intervals so as to fill the chambers within 75 min. After a further 15 min at room temperature the apparatus is placed for 60 min in a water-bath at 27° in the dark for catalyst polymerization. Then a neon lamp is placed perpendicular above the casting chamber. After 3 h of mixed-catalyst photopolymerization the whole box is placed in a refrigerator and the single gradient chambers are dissected from the gel block prior to use.

Stock solutions

A: 36.3 g of Tris, 0.46 ml of N,N,N′,N′-tetramethylethylenediamine, 48 ml of 1 *N* hydrochloric acid, adjustment to pH 8.8, water to 100 ml.

B: 2.8% ammonium peroxodisulphate, freshly prepared 1 h before use and kept at 4°.

CI: 57 g of acrylamide, 3 g of N,N′-methylenebisacrylamide (BIS), water to 100 ml.

CII: 54 g of acrylamide, 6 g of BIS, water to 100 ml.

KI: 80 mg (KII = 800 mg) of $K_3Fe(CN)_6$ per 100 ml (maximum keeping time 4 weeks).

R: 8 mg of riboflavin per 100 ml (maximum keeping time 2 weeks).

G: 50 g of glucose per 100 ml, 0.1% sodium azide.

Slab gradient, T = 3–25%, C = 5%; 3% = 12.5 ml of A, 2.5 ml of B, 5 ml of CI, 7.5 ml of KI, 10.0 ml of R and 62.5 ml of water; 25% = 12.5 ml of A, 2.5 ml of B, 41.6 ml of CI, 7.5 ml of KI and 35.9 ml of G.

Cylindrical gradients

The same type of mixed polymerization was used for the 5-mm cylinder gels. The gradient preparation chamber, assembled from two glass plates (18 × 16 cm for 12 tubes, 18 × 32 cm for 24 tubes) and soft silicone spacer tubing (O.D. = 12 mm, wall thickness 2–2.5 mm) is held together with screw-clamps. The tubes, provided with 3–4-mm Parafilm collars at the upper edge, are held in place by a 12-(or 24-) hole frame which rests on the polymerization chamber. The monomer solutions are pumped into the chamber by a multichannel syringe distribution system as described earlier[18]. All other conditions are identical with those for the slab gradients.

Large-pore cylinder gradient, T = 3–40%, C = 5–10%; 3% = 12.5 ml of A, 2.5 ml of B, 5 ml of CI, 10 ml of KI, 4 ml of R and 66 ml of water; 40% = 12.5 ml of A, 2.5 ml of B, 67 ml of CII, 7.5 ml of KI and 10.5 ml of G.

Small-pore cylinder gradient, T = 20–50%, C = 10%; 20% = 12.5 ml of A.

2.5 ml of B, 33 ml of CII, 0.75 ml of KII, 3.75 ml of R and 47.5 ml of water; 50% = 12.5 ml of A, 2.5 ml of B, 83.0 ml of CII, 0.75 ml of KII and 1.25 ml of G.

Anode buffer (pH 8.5): 28.5 g of Tris, 136.8 g of glycine, water to 5000 ml (2 l per electrode chamber).

Cathode buffer (pH 8.5): 36.0 g of Tris, 172.8 g of glycine, water to 5000 ml (1 l per electrode vessel).

Sample volume: 0.2–0.5 ml, loaded with 50% glucose, 1 μl of bromophenol red.

Running conditions (constant voltage): 1st day, 75 V; 2nd and 3rd days, 150 V; 4th day, 175 V; 5th day, 225 V.

The buffer is replaced after 12-h intervals. The glass tubes are carefully cracked to liberate the gel cylinders.

Calibration mixtures

The following three protein mixtures were finally adopted for the large-pore cylinder gradients, T = 3–40% (Fig. 4).

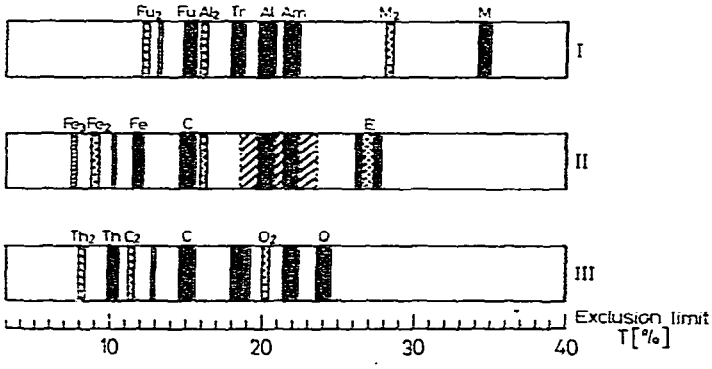


Fig. 4. Band patterns of the calibration mixtures for the large-pore cylinder gradients. The exclusion limits indicated are average values from numerous linear gradient experiments. Al = albumin; Am = α-amylase; C = catalase; E = egg trypsin inhibitor; Fe = ferritin; Fu = fumarase; M = myoglobin; O = ovalbumin; Th = thyroglobulin; Tr = transferrin.

I. 75 μg of whale myoglobin, carbamylated (Schuchardt, Darmstadt, G.F.R.), 150 μg of *Bacillus subtilis* α-amylase (Serva), 50 μg of human serum albumin (Serva), 50 μg of human serum transferrin (Behring-Werke, Marburg, G.F.R.) and 70 μg (35 μl) of pig heart fumarase (Boehringer, Mannheim, G.F.R.).

II. 200 μg of egg trypsin inhibitor, molecular weight 28,000 (Serva), 60 μg (3 μl) of bovine liver catalase (Boehringer), 145 μg (2 μl) of horse spleen ferritin (Miles, Kankakee, Ill., U.S.A.) and amylase and albumin as above.

III. 70 μg of ovalbumin (Serva), 450 μg of bovine thyroglobin (Sigma, St. Louis, Mo., U.S.A.) and amylase, transferrin and catalase as above.

The proteins are dissolved in 0.1 ml of saline. Human serum can be used as a supplementary reference mixture (Fig. 5). Some proteins (myoglobin, ribonuclease and insulin) do not approach their exclusion limits unless they have been acidified by thorough carbamylation in 2 M KCNO at 37° for 3 h. The reaction mixtures (50–100 μl) can be immediately applied together with the other reference proteins on to the gel gradient.

Fig. 5. Band pattern of human serum on large-pore cylinder gradients. The following proteins are easy to identify and can be used for calibration purposes: PA = prealbumin; Alb = albumin; Tf = transferrin; α_2M = α_2-macroglobulin; βLP = β-lipoprotein.

Calibration mixtures for the small-pore cylinder gradients, $T = 20$–50%, were as follows (Figs. 6 and 7).

(I) 100 μg of bovine insulin B-chain, molecular weight 3400 (Boehringer), 100 μg of ribonuclease A, carbamylated (Boehringer) and 50 μg of human serum albumin (Serva).

(II) 100 μg of β-lactoglobulin, molecular weight of the monomer = 17,900 (Serva), 50 μg of human serum transferrin (Behring-Werke) and insulin B-chain as above.

(III) 200 μg of insulin, carbamylated, molecular weight = 5700 (Serva), 100 μg of soya bean trypsin inhibitor, molecular weight = 21,500 (Serva) and 150 μg of egg trypsin inhibitor, molecular weight = 28,000 (Serva).

(IV) 300 μg of parathyroid hormone, molecular weight = 4100 (Beckman, Palo Alto, Calif., U.S.A.), 50 μg of whale myoglobin, carbamylated (Schuchardt) and 200 μg of acid α_1-glycoprotein, molecular weight = 44,100 (Behring-Werke).

SDS electrophoresis in the second dimension

Immediately after the first-dimensional run, the agarose strips are immersed in

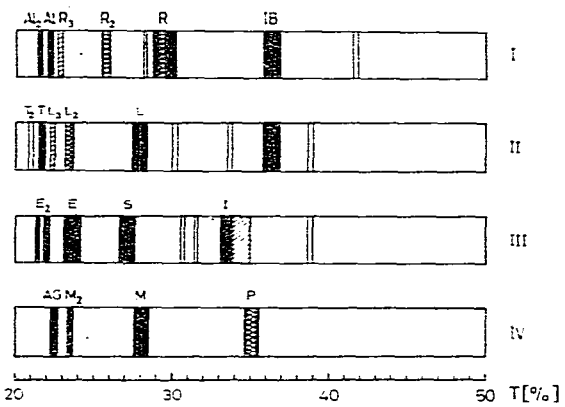


Fig. 6. Band patterns of the calibration mixtures for the small-pore cylinder gradients. The exclusion limits indicated are average values from eight linear gradient experiments. AG = acid α_1-glycoprotein; Al = albumin; E = egg trypsin inhibitor; I = insulin; IB = insulin B-chain; L = lactoglobulin; M = whale myoglobin; P = parathyroid hormone; R = ribonuclease; S = soya bean trypsin inhibitor; T = transferrin.

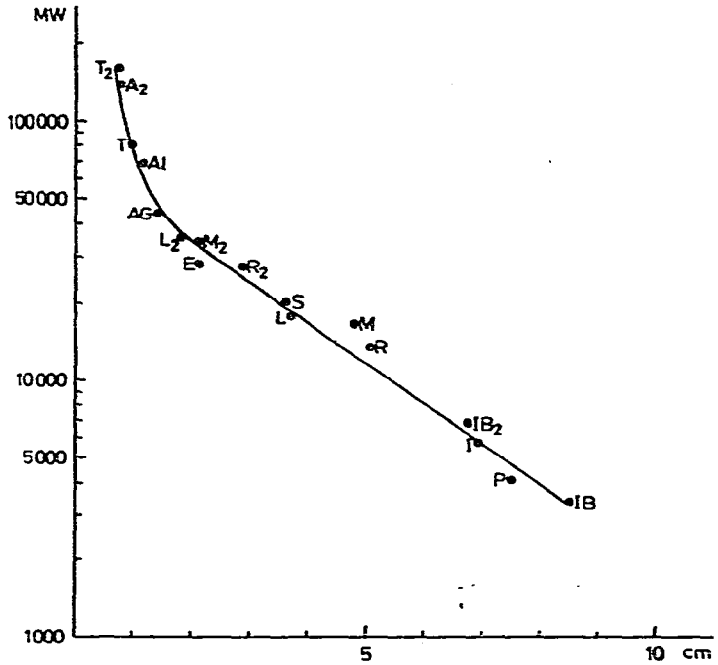


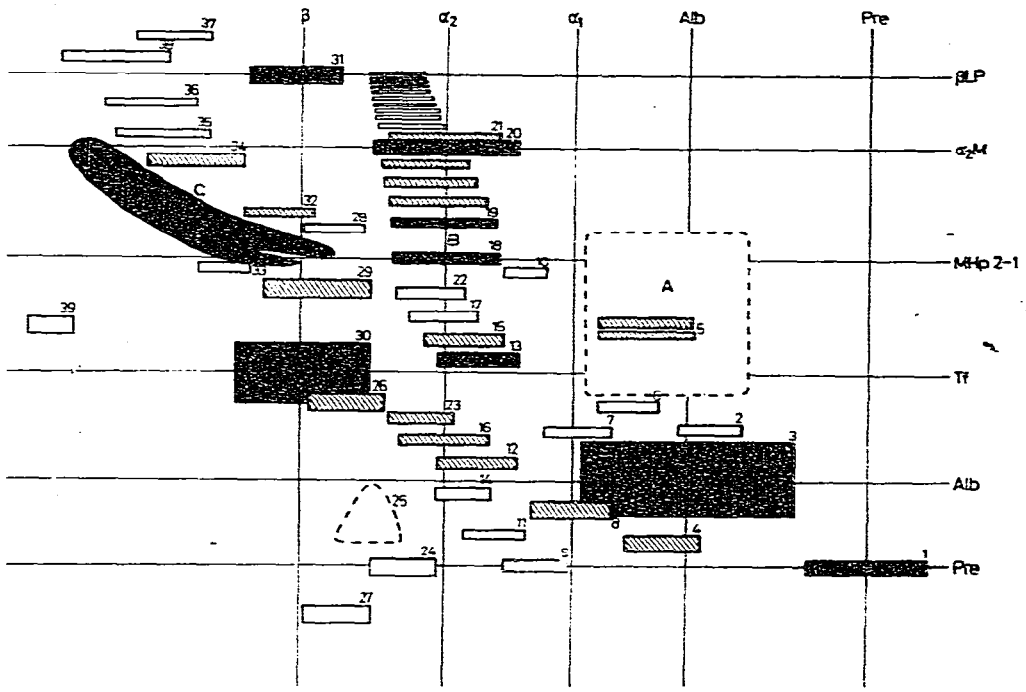
Fig. 7. Representative calibration graph for cylindrical gel gradients, $T = 20$–50%, obtained with the four calibration mixtures in Fig. 6.

1% SDS for 20 min at 45°. Separation in the second dimension can be performed either on isoporous gel slabs or on 3–25% gradients. As expected, the proteins of γ-mobility now migrate according to molecular weight. The strongly charged heterogeneous immunoglobulin family, which appears as a broad comma-like zone in the native state (Fig. 2), now forms a narrow band. The SDS treatment is not drastic enough to dissociate all composite reference and serum proteins, *e.g.*, the tetrameric prealbumin is only partially dissociated into the half-molecule, and both catalase and ferritin remain completely intact. Most oligomers, however, dissociate (*e.g.*, albumin), and some proteins vanish completely, *e.g.*, the high-density lipoproteins.

DISCUSSION

Two-dimensional techniques[19–27] that are able to single out the majority of individual constituents from a complex mixture of soluble proteins without the addition of extraneous substances such as urea, detergents or polymeric zwitterions, are attractive alternative analytical tools. Well known protein mixtures, *e.g.*, human serum, should be applied to compare the capacity and reliability of salient high-resolution techniques. The detection sensitivity of the two-dimensional agarose/PA gradient technique is *ca.* 1 μg with Coomassie blue staining, corresponding to a single protein level of about 10 mg per 100 ml. There are at least 31 thoroughly studied serum proteins with levels above 10 mg per 100 ml and it is unlikely that the number will increase substantially in the future. Twenty of these proteins have been localized, and there are a further seventeen well marked spots, which have not been or

have only tentatively been identified (see Fig. 8). Apart from these 37 components there are the oligomers of albumin and immunoglobulin G, the variable subfractions of the high-density lipoproteins (3–5) and the haptoglobin polymers (up to 15). It is unrealistic to expect more than 50–60 individual protein bands after conventional staining if one adheres to the "one spot–one protein" condition. The technique is virtually free of hazards, at least on application of serum. There is neither a precipitate left in the agarose gel nor a macromolecular aggregate detectable in the large-pore polyacrylamide gel, and there is no streak visible from the application slit downwards. Several other human body fluids have been tested, but none of them is more complex than serum, whether the fluid is primarily a product of filtration (*e.g.*, urine, cerebrospinal fluid, amniotic fluid, synovial fluid, cyst fluid) or secretion (*e.g.*, tears, colostrum,



1 Prealbumin
2 ?
3 Albumin
4 Acid α_1-Glycoprotein
5 Albumin-Dimer
6 α_1T-Glycoprotein?
7 α_1-Antichymotrypsin
8 α_1-Antitrypsin
9 ?
10 Inter α_1-Trypsininhibitor?
11 3,8 S α_2-Glycoprotein?
12 Gc-Globulin
13 Haptoglobin 1-1
14 Zn-α_2-Glycoprotein?
15 Ceruloplasmin
16 α_2HS-Glycoprotein
17 ?
18 Monomeric Haptoglobin 2-1
19 Monomeric Haptoglobin 2-2
20 α_2-Macroglobulin
21 ?
22 ?
23 ?
24 β_2-Glycoprotein I?
25 ?
26 Hemopexin
27 β_2-Glycoprotein III?
28 ?
29 β_1A-Globulin
30 Transferrin
31 Low Density Lipoprotein
32 β_1C-Globulin
33 Plasminogen?
34 IgG-Dimer
35 IgG-Trimer
36 IgG-Tetramer
37 ?
38 Immunoglobulin M
39 ?

A High Density Lipoproteins
B Haptoglobin-Polymers
C Immunoglobulins A+G

Fig. 8. Schematic presentation of the normal two-dimensional serum pattern. The following proteins were chosen for constructing the charge/size network: prealbumin, albumin, α_1-antitrypsin, α_2-macroglobulin, β-transferrin and monomeric haptoglobin 2-1 and β-lipoprotein. The variable subfractions of the high-density lipoproteins have been omitted.

milk, seminal plasma). It remains to be seen whether extracts of soluble tissue proteins are more complex than serum.

If isoelectric gel focusing instead of electrophoresis is performed in the first dimension one may separate several hundred spots on application of serum[28], but it seems almost impossible to differentiate microheterogeneities from individual proteins. This is especially true for the numerous spots in the region of the medium-sized α_1/α_2-globulins. Some proteins, *e.g.*, transferrin, may separate into at least twenty charge and size heterogeneous spots, and it is difficult to interpret the frequent pearl necklace appearance of certain bands.

As the minimal resolving capacity on application of serum proteins is ten bands in the first (agarose) and twenty bands in the second dimension (gradient), one should be able to single out 100–200 individual components under optimal conditions. Serum proteins do not distribute evenly over the entire slab area. There are virtually no fast-moving high-molecular-weight proteins and only a few slow-moving small proteins, *e.g.*, γ-trace protein and the β_2-glycoproteins.

The most favourable two-dimensional technique would exploit "pure" molecular properties as isoelectric points, free electrophoretic mobilities, hydrodynamic sizes or molecular weights to obtain a two-axial grid over which the individual components can distribute statistically. Only then is each individual running position equivalent for a twin set of molecular parameters. Electrophoresis on restrictive media, *e.g.*, polyacrylamide gel[19,24], is unsuitable for this purpose as the running position depends on both size and charge. Even low-restrictive polyacrylamide gels prevent the attainment of a focusing equilibrium for the largest serum proteins, *e.g.*, α_2-macroglobulin, IgM and β-lipoprotein[24,29].

The resolving power of various electrophoretic techniques is generally evaluated by subjective standards, and it is desirable to develop some kind of objective parameter to compare various methods. One can use the maximal number of constituents that are clearly discernible within a certain migration path as a provisional criterion if a universally accepted reference mixture, *e.g.*, freshly obtained serum, preferentially of Hp-type 2-1, is applied. By this standard the discontinuous Ornstein–Davis polyacrylamide electrophoresis resolves 28 defined individual serum proteins with an average density of five bands per centimetre on 6.5% gel. The maximal band density decreases with the migration distance from 8 per centimetre in the macroglobulin region to 3 per centimetre in the region of medium-sized α-globulins.

The validity of the running position on gel gradients as a size parameter is based on the premise that the exclusion limit is reached or at least closely approached after long running times. The exclusion limit, as determined by a series of graded homogeneous polyacrylamide gels, can be correlated with the hydrodynamic size of proteins that have an overall globular shape[9]. For this group of "normal" proteins, the molecular weight can be estimated[30–32] provided that the frictional ratio is within the normal range. To give an example: the hydrodynamic volume of the very voluminous but globular immunoglobulin M molecule can be reliably evaluated by exclusion electrophoresis, but the molecular weight is considerably overestimated for its very space-occupying polyp-like structure[33], which explains the unusual high frictional ratio ($R/\bar{R} = 1.97$). Extended molecules such as fibrinogen are characterized by large frictional ($R/\bar{R} = 2.34$) *and* axial ratios (longest axis/shortest axis (a/c) = 7.3), but the error is reduced by molecular alignment during migration through the gel meshwork[9].

The separation principle of gel exclusion (or pore limit) electrophoresis contradicts the postulate that exact molecular weights of native proteins can be determined on gradients. As in partition chromatography, the proteins have to be denatured by urea, guanidine or SDS to eliminate differences in conformation and hydration. Therefore, SDS and exclusion electrophoresis cannot compete in molecular weight determinations, but the latter technique is accurate enough for evaluating the number of SDS-initiated subunits that comprise the native globular protein.

Similarly, the exclusion limit is a more relevant molecular parameter if a separation strategy has to be devised for a particular protein, as both partition and retardation coefficients depend primarily on the hydrodynamic volume and not on the molecular weight. The permeability behaviour of proteins *in vivo* is also controlled by hydrodynamic properties and not the molecular mass[34]. For all practical purposes, however, the molecular weight can be used as an operational size parameter for globular proteins if one considers the underlying restrictions.

The experimental determination of exclusion limits is rather cumbersome and can be replaced with sufficiently long running times on polyacrylamide gradients to obtain an equivalent experimental value. However, proteins of γ-mobility (p$I > 7$) still do not move fast enough to reach their pore limits within reasonable running times. As all standard proteins selected for the two-dimensional technique migrate faster than β-transferrin, proteins such as myoglobin, γ- and β-trace protein or the immunoglobulins cannot be evaluated accurately. This, however, can be overcome if charge differences are wiped out by SDS in the second dimension. In addition, the $T = 3$–25% gradients can still be used for proteins of molecular weight above 30,000, and homogeneous 20% gel slabs are appropriate for the range 5000–30,000 daltons. Even very alkaline proteins such as ribonuclease and cytochrome *c* can then be used as molecular weight markers.

Unfortunately, agarose gel cannot be heated to 100° and the comparatively mild conditions used (30 min at 45°) produce erratic effects on the individual components of a soluble protein mixture, which can be evaluated, however, by a band to band comparison of both patterns. Some proteins remain completely intact (*e.g.*, ferritin), some dissociate partially (*e.g.*, prealbumin) and others strongly. Some body fluids, *e.g.*, urine or milk, contain considerable proportions of low-molecular-weight proteins below 40,000 daltons that have to be differentiated from SDS-initiated subunits of larger molecules. If the nature of each spot has been established by comparison of the two-dimensional patterns, either the cylindrical small-pore $T = 20$–50% gradient or standard SDS electrophoresis may be the method of choice for further examinations.

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