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THIN-LAYER GEL FILTRATION OF PROTEINS

II. APPLICATIONS

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SUMMARY

Human and animal serum proteins have been separated into three fractions on Sephadex G-200. The percentage contents of these fractions in human serum were estimated after densitometric evaluation.

Glycoproteins of horse and human serum have been separated on Bio-Gel P-300 thin layers and detected by periodic acid–Schiff staining.

Soluble proteins from mammalian cells grown *in vitro* (normal human fibroblasts and HeLa cancer cells) have been separated on Sephadex G-200 into six fractions. The quantitative contents and the molecular weights of these fractions have been determined.

Five fractions have been detected in soluble leaf proteins from spinach, the molecular weights ranging from 18 600 to more than 700 000.

The molecular weight of *o*-diphenol oxidase from mushrooms was 50 000 for a mixture of polyphenols and tyrosine used as a substrate. In addition to this enzyme, in mushrooms with an open cap another enzyme appeared with a molecular weight of 115 000. The molecular weight of a commercial tyrosinase preparation was 135 000.

In the previous paper several aspects of thin-layer gel filtration of proteins were examined and an improved method was described[1]. Linear relationships between migration, referred to a standard protein (myoglobin), and molecular weight or Stokes radius of a number of well defined globular proteins were established, thus providing a basis for estimation of these size parameters. Application of the sample as a streak and subsequent densitometric evaluation of the pattern has made possible a quantitative approach which was considered to be of value in analyzing heterogenous protein systems. In the present paper some applications of this modified method are described, including separation of protein systems of both animal and plant origin. In addition to separation of serum proteins on Sephadex G-200, separation of serum glycoproteins in thin layers of the polyacrylamide Bio-Gel P-300 is also described. The usefulness of thin-layer gel filtration for the rapid determination of the molecular weight of enzymes is illustrated by the results obtained with mushroom *o*-diphenol oxidase (EC 1.10.3.1).

EXPERIMENTAL

Materials

Sephadex gel G-200 "superfine" was obtained from Pharmacia, Uppsala, Sweden. The polyacrylamide gel Bio-Gel P-300 came from Bio-Rad Laboratories, Richmond, Calif., U.S.A. Myoglobin was supplied by Koch and Light, Colnbrook, England and tyrosinase was obtained from Sigma, St. Louis, U.S.A. Human and animal sera were used without added preservatives within a few days after preparation.

METHODS

Preparation of the plates, application of the sample, separation, staining and densitometric evaluation were carried out as described in the previous paper[1].

Protein-bound carbohydrates

Protein-bound carbohydrates were detected by the periodic acid–Schiff reaction (PAS reaction). The Schiff reagent[2] was diluted before use to 1:1, v/v with 95% ethanol[3]. The stained strips were washed in a $NaHSO_3$ solution acidified with HCl and subsequently with acetone to which 1% trichloroacetic acid had been added[4]. For densitometry a 550 filter was used.

Soluble proteins from mammalian cells grown in vitro

HeLa cells and human fibroblasts after 10–20 passages (Institute of Histology, University of Vienna) were cultivated as monolayers either in a lactalbumin hydrolysate medium (HeLa) or in Parker's medium 199 (fibroblasts) supplement by 5% chicken embryonic extract and 10–20% human umbilical cord serum with the addition of antibiotics[5]. The cells were detached from the glass by EDTA treatment, washed four times with 0.9% NaCl solution containing 0.02% KCl and $CaCl_2$. Before use the washed cells were kept as a sediment at −30°. The cells (50–200 × 10^6) were homogenized in a Potter–Elvehjem homogenizer, in 1–3 ml 0.02 *M* phosphate buffer (pH 7.2–7.4), supplemented by three freeze-thawing cycles at −30°. The homogenate was centrifuged at 34 000 *g* for 15 min and the supernatant concentrated by evaporation *in vacuo* to about 2–3% protein concentration.

Leaf proteins

Leaf proteins were isolated from spinach. *Step 1*: Ten grams of fresh spinach leaves were homogenized with 50 ml of a 4 *M* $(NH_4)_2SO_4$ solution in 0.05 *M* phosphate buffer (pH 7.2–7.4) in a MSE homogenizer operated at full speed for 3 min, the vessel being cooled in an ice bath. The resulting suspension of protein precipitate and cell debris was filtered through a Büchner funnel and the precipitate washed on the filter with 4 *M* $(NH_4)_2SO_4$ solution. *Step 2*: After washing, the precipitate was extracted with 0.1 *M* phosphate buffer (pH 7.2–7.4) and centrifuged at 34 000 *g* for 15 min. The sediment was once again extracted with 0.1 *M* phosphate buffer and centrifuged as above. *Step 3*: The supernatants from both centrifugations were combined and the soluble proteins salted out with ammonium sulphate (70 g/100 ml). The protein precipitate was separated by high-speed centrifugation and the sediment obtained was dissolved in 0.1 *M* phosphate buffer (pH 7.2–7.4). The protein solutions were used

in 2–4% concentration. Sometimes the solutions were found to have an exceptionally high viscosity, but this could be reduced by repeating Step 3.

o-Diphenol oxidase

Common cultivated mushrooms, either fresh or frozen at −20°, were employed as a starting material for the preparation of *o*-diphenol oxidase. In most experiments a single mushroom was used. It was homogenized at 2–4° in a porcelain mortar for 3–5 min with an equal volume of pre-chilled, deaerated 0.1 *M* phosphate buffer, pH 7.2–7.4. In some preparations ascorbic acid was added to the phosphate buffer to obtain a final concentration of 50 m*M* in the homogenate. The homogenates were centrifuged immediately in the cold at 34 000 *g* for 15 min. After centrifugation, 10–20 μl of the clear, yellow supernatant were placed without delay on a Sephadex G-200 plate with myoglobin as a standard in the middle. After a run of 5–7 h, a print was taken with Whatman No. 3 paper impregnated with a mixture of polyphenols and tyrosine (mixed substrate) or with tyrosine alone. The mixed substrate, prepared in deaerated 0.02 *M* buffer phosphate pH 7.2–7.4, contained caffeic acid (0.0015 *M*), DOPA (3,4-dihydroxyphenylalanine, 0.0015 *M*), pyrocatechol (0.01 *M*), pyrogallol (0.01 *M*) and tyrosine (0.0015 *M*); the latter was also used alone at the same concentration. The paper was dipped into the substrate and was subsequently dried at room temperature. To detect enzyme activity, the paper was left in contact with the gel for about 5–10 min, then removed from the plate and dried at room temperature. Maximum colour usually developed after 20 min. A decrease in colour was observed during the next few hours; the remaining colour was stable.

RESULTS

Serum proteins

A separation on Sephadex G-200 of normal serum proteins from pooled human and animal sera is shown in Fig. 1. Three fractions can be clearly distinguished in both the human and the animal sera, corresponding to the three principal classes of proteins with sedimentation constants of 4*s*, 7*s* and 19*s*. Densitometric tracings of two of the sera are shown in Fig. 2.

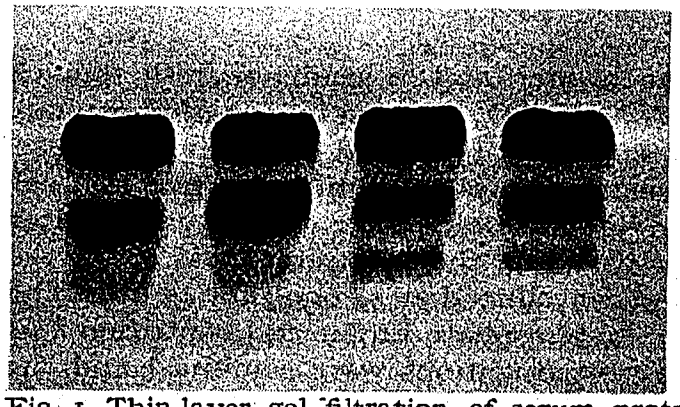

Fig. 1. Thin-layer gel filtration of serum proteins on Sephadex G-200. Sample: 10 μl of undiluted serum proteins. Plate 20 × 20 cm. Separation time: 6 h. Staining with Amido Black 10B. From left to right: human, horse, pig and bovine serum. From top to bottom: 4*s*, 7*s* and 19*s* components of serum.

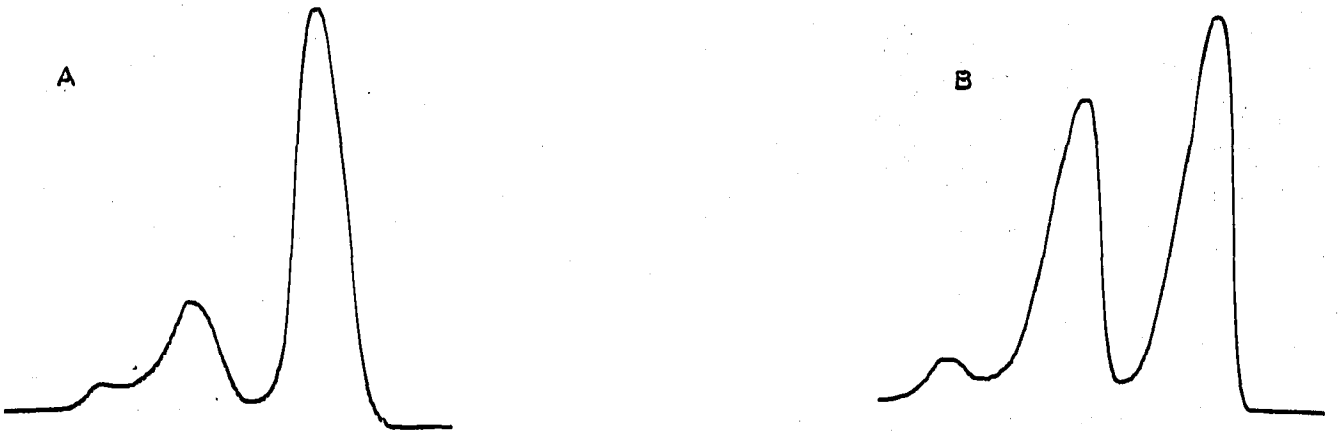

Fig. 2. Densitograms of (A)-human and (B)-horse serum proteins separated on Sephadex G-200 From left to right: 19*s*, 7*s* and 4*s* serum components.

While all the sera were qualitatively similar, distinct quantitative differences in the pattern of human and horse serum can easily be seen in the content of the 7*s* fraction.

The R_M values for the three fractions were determined in experiments using the method of internal standard. The sera were diluted with 0.5 *M* NaCl containing 0.02 *M* phosphate buffer (pH 7.2–7.4), myoglobin being added to a final concentration of 1%. Ten microliters of the mixture were applied to the plate. In Table I the R_M values measured on the densitometric tracings for the maxima of the peaks are shown. Included also are the percentage contents obtained on planimetry of the area in the densitogram for several dilutions, after staining with Coomassie Blue and Amido Black. The latter values were corrected for incomplete removal of albumin by the print[1].

The periodic acid–Schiff staining method (PAS reaction) cannot be applied to prints of Sephadex thin layers because of heavy background staining. As an alternative, thin-layer gel filtration on the polyacrylamide Bio-Gel was tried and found to be suitable. Two preparations of Bio-Gel P-60 and P-300 having different optimal fractionation ranges were employed. With the latter, serum glycoproteins were separated into three to four zones (Figs. 3 and 4). In order to compare the glycoprotein

TABLE I

R_M VALUES AND PERCENTAGE CONTENTS OF HUMAN SERUM PROTEINS SEPARATED BY THIN-LAYER GEL FILTRATION ON SEPHADEX G-200

Component	R_M *value*	*Serum dilution*[a]					
		Coomassie Blue			*Amido Black 10 B*		
		1 + 4	*1 + 6*	*1 + 8*	*1 + 2*	*1 + 3*	*1 + 4*
4*s*	1.49	77.9	76.8	84.6	70.5	75.9	78.1
7*s*	1.99	20.2	21.3	13.8	25.9	21.0	19.7
19*s*	2.35	1.9	1.9	1.5	3.6	3.1	2.2

[a] Dilution with 0.5 *M* NaCl + 0.02 *M* phosphate buffer, pH 7.2–7.6.

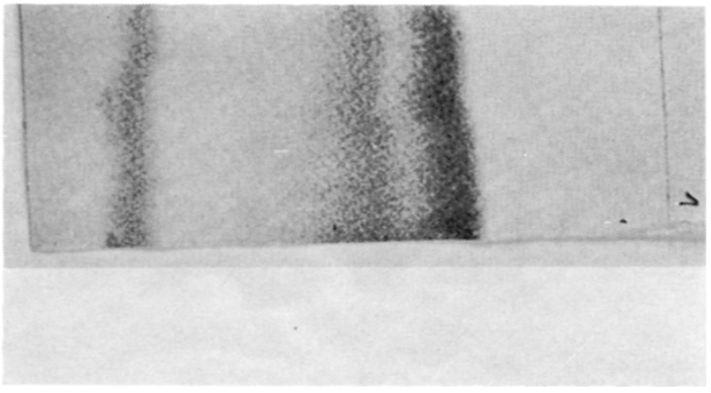

Fig. 3. Thin-layer gel filtration of glycoproteins on Bio-Gel P-300. Sample: 10 μl per cm of undiluted horse serum. Periodic acid–Schiff staining. From left to right: 19*s*, 7*s* and 4*s* components.

Fig. 4. Densitogram of horse serum glycoproteins separated on Bio-Gel P-300.

pattern with that of the proteins, 70 μl of undiluted serum were applied by means of a microscopic slide (length 7.5 cm) to a 20 × 20 cm plate, two samples being run onto one plate. After a run of 25–30 h, a print was taken with filter paper Whatman No. 3 and dried as usual at 110°. Afterwards the print was cut lengthwise along each separated sample and one half was stained with Amido Black for protein detection; the other half was stained by the PAS reaction. The protein pattern was very similar to that obtained on Sephadex G-200. Three zones corresponding to the 4*s*, 7*s* and 19*s* components were detected. The zone of the 7*s* fraction, however, was broader. It was composed of a weakly stained leading fraction and a strongly stained fraction migrating more slowly. PAS staining also revealed three to four zones in human and animal sera. Quite a considerable fraction of the PAS-stained material resides in the 19*s* fraction. The PAS-stained material parallels that stained with Amido Black for protein detection; the relative distribution, obtained with both methods, however, is quite different.

Soluble proteins of mammalian cells grown in vitro

Soluble proteins were isolated from normal human fibroblasts and HeLa cancer cells, the 34 000 *g* supernatant being used for the gel filtration experiments. Since rather limited amounts of cells grown *in vitro* are available, Coomassie Blue was tried as a stain and found to be much superior to Amido Black due to its increased sensitivity. When fibroblast and HeLa soluble proteins were separated on Sephadex G-200 on the same plates, a complex pattern of six fractions was observed (Figs. 5 and 6). There

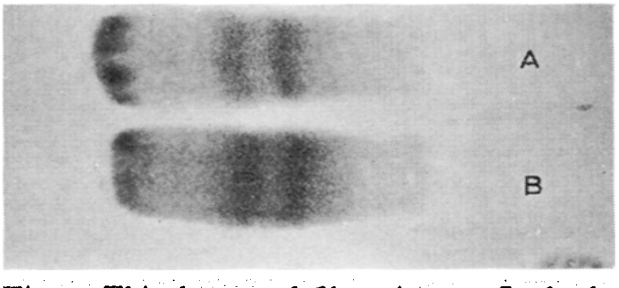

Fig. 5. Thin-layer gel filtration on Sephadex G-200 of soluble proteins from (A) normal human fibroblasts and (B) HeLa cancer cells. Staining with Coomassie Blue.

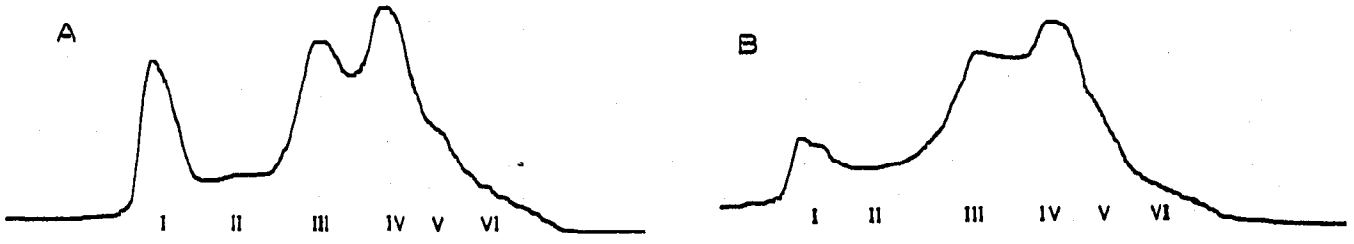

Fig. 6. Densitograms of soluble proteins from (A) normal human fibroblasts and (B) HeLa cancer cells (see Fig. 5).

was a basic similarity in both patterns, but quantitative differences in the contents of different fractions can easily be noted in the densitometric tracings. The R_M values for the particular fractions were determined from the densitograms by extrapolating the peaks to Gaussian curves and drawing a perpendicular from the top of the peak to the base line. The distance of this perpendicular from the starting line was measured and divided by the distance for myoglobin similarly obtained. In this way, R_M values for the weak fractions can be estimated with greater accuracy than by direct measurement on the plate. For the weakest fractions, measurements directly on the plate become impossible. The R_M values, the corresponding molecular weights[1] and the quantitative percentage contents of these fractions obtained in eight runs of each material are shown in Table II. The percentage contents of fractions III to VI were corrected for incomplete removal by the print technique as described previously[1].

TABLE II

PERCENTAGE CONTENTS, R_M VALUES AND MOLECULAR WEIGHTS OF SOLUBLE PROTEINS FROM MAMMALIAN CELLS GROWN *in vitro* AFTER THIN-LAYER GEL FILTRATION ON SEPHADEX G-200

Fraction	*Fibroblasts*			*HeLa cells*		
	% content	*R_M value*	*Molecular weight*	*% content*	*R_M value*	*Molecular weight*
I	17.6	2.36	⩾600,000	11.5	2.35	⩾600,000
II	8.5	2.02	240,000	9.6	2.10	300,000
III	28.8	1.72	107,000	32.1	1.76	120,000
IV	31.4	1.48	56,000	30.4	1.50	59,000
V	9.4	1.26	31,000	11.6	1.26	31,000
VI	4.3	1.09	19,600	4.8	1.09	19,600

Soluble plant proteins

Soluble leaf proteins from spinach leaves could be separated into five fractions (Fig. 7). The R_M values of these fractions after staining with Coomassie Blue were determined from the densitometric tracings after extrapolation to Gaussian curves as described above for the soluble proteins derived from mammalian cells. The molecular weights of these fractions are shown in Table III together with their percentage contents corrected for incomplete removal by the first print as described previously[1]. A few experiments were performed with soluble proteins isolated from potato tubers. Again a complex pattern of three to four fractions was observed.

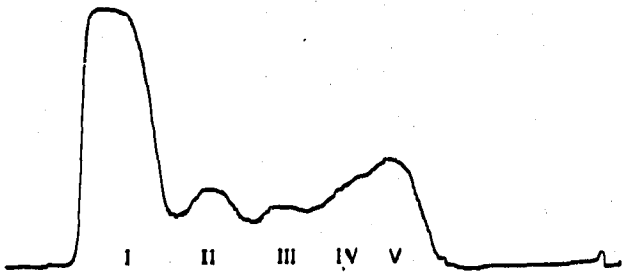

Fig. 7. Soluble leaf proteins from spinach separated by thin-layer gel filtration on Sephadex G-200. Densitogram after staining with Coomassie Blue.

TABLE III

PERCENTAGE CONTENTS, R_M VALUES AND MOLECULAR WEIGHTS OF SOLUBLE LEAF PROTEINS SEPARATED BY THIN-LAYER GEL FILTRATION ON SEPHADEX G-200

Fraction	*% content*	*R_M value*	*Molecular weight*
I	38.1	2.42	⩾700,000
II	14.0	1.95	200,000
III	11.3	1.62	82,000
IV	10.8	1.36	40,000
V	25.8	1.07	18,600

Enzymes

Thin-layer gel filtration has been used to determine the molecular weight of *o*-diphenol oxidase (EC 1.10.3.1) from mushrooms. A homogenate prepared from mushrooms frozen at −20°, as described under Methods, contained enough enzyme activity to be easily detected on the gel. The homogenate was applied to Sephadex G-200 plates immediately after its preparation to avoid any storage effect. After a run of 4–7 h, a print was taken with a dry filter paper Whatman No. 3 impregnated previously with a mixture of polyphenols containing caffeic acid, DOPA, pyrocatechol, pyrogallol and tyrosine. Alternatively a paper treated with tyrosine only was used.

The substrate-impregnated paper was left in contact with the gel for about 5–10 min. An intensive reaction could be observed within well defined symmetric zones comparable in size to those of standard proteins after staining with Amido Black (Fig. 8). The distances of the enzyme zones from the starting line were measured directly on the plates, as well as the distance of myoglobin, which was sufficiently distinct to be evaluated without staining. The R_M values obtained for a number of different preparations were 1.42, corresponding to a molecular weight of ~50 000. There was no difference in the R_M values obtained with mixed substrate or with tyrosine.

Homogenates prepared in the presence of ascorbic acid sometimes gave qualitatively different pictures from those prepared in its absence. The ascorbic acid homogenates contained, in addition to the R_M 1.42 component, a second component visible as a more intensive narrow zone (3–4 mm broad) in the trailing part of the main zone. This zone could be detected both with the mixed substrate and with tyrosine. The R_M value obtained was 1.30, corresponding to a molecular weight of 35 000.

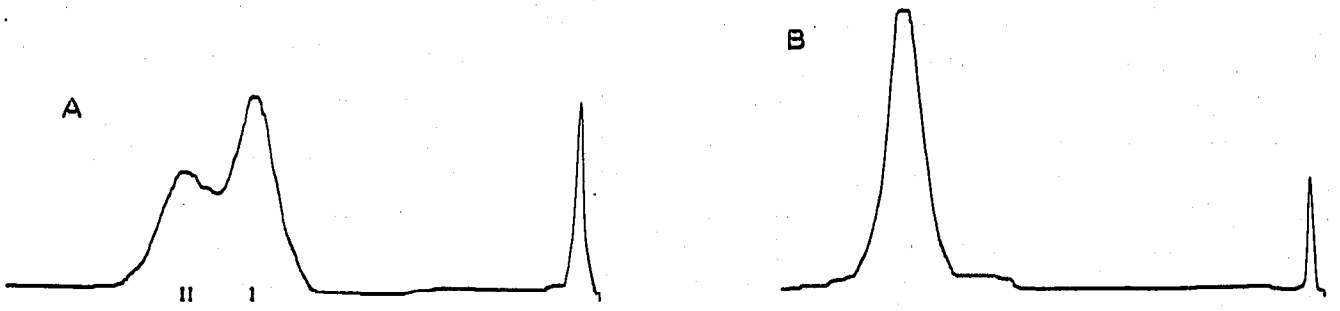

Fig. 8. *o*-Diphenol oxidase separated by Sephadex G-200 thin-layer gel filtration. Densitogram of a print taken with paper impregnated with a mixed substrate containing caffeic acid, DOPA, pyrocatechol, pyrogallol and tyrosine. A — Homogenate of a mushroom with an open cap; B — commercial "tyrosinase". Molecular weight (I) ~50 000; (II) ~115 000. Peaks on right: starting line.

Homogenates prepared without or with the addition of ascorbic acid and stored overnight at 4° in a closed vessel without access of air, showed an appreciable loss of activity, but the R_M values remained unchanged. Salting out of the proteins from the freshly prepared homogenate with ammonium sulphate at 4° at a saturation of 0.66, or precipitation with acetone at −30° and subsequent redissolving of the precipitate to the original volume of the homogenate, had no influence on the R_M value. However, heating the homogenate at 60–65° for a few minutes yielded preparations which could be resolved by thin-layer gel filtration into two zones of activity. In addition to the zone present in the untreated homogenate a component with a higher R_M value was also observed.

With a commercial preparation of "tyrosinase" (Sigma Chemical Company, St. Louis, U.S.A.) only one zone of activity was observed with an R_M value of 1.80, both for the mixed substrate and tyrosine, corresponding to a molecular weight of 135 000, which is much higher than that obtained with the freshly prepared homogenates.

Using mushrooms of differing maturity, however, a striking difference was observed. Mushrooms with a closed cap showed only one zone of enzyme activity with an R_M value corresponding to the 50 000 molecule, whereas homogenates of mushrooms with a cap already open yielded two zones of activity, one corresponding to the 50 000 molecule, the other with an R_M value of 1.75, corresponding to a molecular weight of 115 000 (Fig. 8A).

DISCUSSION

Some of the applications of thin-layer gel filtration described in this paper serve to emphasize the versatility of the method. Separation of serum proteins using gel columns has become an established procedure, and it was also the object of early applications of thin-layer gel filtration[6–10]. A simple and reproducible method of separating serum proteins should be of considerable clinical importance. Three fractions could be distinguished in both human and animal sera corresponding to the three principal classes of proteins having the sedimentation constants 4*s*, 7*s* and 19*s*. The assignment of the components to ultracentrifugal classes has been demonstrated in column gel filtration experiments[11].

The R_M value determined for the 4*s* fraction on thin-layer gel filtration differs from that of isolated albumin[1], the discrepancy probably being due to the heterogeneity of this peak. Albumin is the main component of the 4*s* peak, but it is accompanied by other components differing in molecular weight[12]. For the 19*s* fraction an R_M value of 2.35 would correspond to a molecular weight of about 600 000, assuming that the R_M value–molecular weight relationship in this range is still linear. It has been pointed out, however, that there is insufficient evidence for linearity of the R_M value–molecular weight relationship in the range of molecular weights above 240 000 (see ref. 1).

The percentage contents of the three classes of serum proteins, after planimetric evaluation, clearly depend on dilution of the serum (Table I). With increasing protein concentration, the values for the 4*s* fraction decrease. For some of the dilutions, the percentage contents are fairly constant with both of the dyes tested. Depending on the aim of the analysis two dilutions will probably yield more accurate values for the 4*s*/7*s* and 7*s*/19*s* ratios than can be obtained with only one dilution. The values for the percentage contents of the three classes of serum proteins obtained on thin-layer gel filtration with subsequent densitometric evaluation are very similar to those obtained by elution of proteins stained with Amido Black from a paper print[9], the main difference being in the values for the 19*s* fraction.

The data on the quantitative distribution of the three classes of serum proteins in thin-layer gel filtration (Table I) may be compared with those obtained on ultracentrifugation. For the 4*s*, 7*s* and 19*s*, peak percentages of 83–86, 11–13 and 3–4, respectively, are accepted[12,13]; however, values which are lower for the 4*s* and higher for the 7*s* components were obtained in recent ultracentrifugal estimations[14,15].

The densitogram of the stained serum proteins separated by thin-layer gel filtration is distinct from the pattern obtained by column gel filtration. The observed difference is due to the different detection methods. In column gel filtration, proteins are detected either by ultraviolet absorption at 280 mμ or shorter wavelengths with some of the flow analyzers, or by the Folin reaction. With all these methods, the results depend strongly on the amino acid content of the proteins. Great differences in the specific extinction coefficients for the three classes of serum proteins have been demonstrated[14].

Gel filtration has already been successfully applied to the analysis of paraproteins[14,16–18]. Recently characteristic column gel filtration patterns for a number of pathological conditions have been described and their value for differential diagnosis stressed[19]. The usefulness of thin-layer gel filtration for the separation of pathologic sera has already been demonstrated[6,7,9,20]. The method described here should provide a basis for a simplified quantitative approach to the analysis of abnormal serum proteins differing in molecular size. Due to the small amount of material needed for analysis, some of the protein-poor body fluids, in addition to serum proteins, could be easily analyzed by this method.

While lipoprotein detection has been successfully performed after thin-layer gel filtration of serum proteins[6], there are no reports on the separation of glycoproteins. This is probably due to technical difficulties with Sephadex gels. Thin-layer gel filtration on Bio-Gel P-300 proved to be a useful approach. The results obtained by this method are in agreement with the physicochemical characteristics of some of the major serum glycoproteins[12]. In view of the importance of glycoprotein analysis for

clinical diagnosis, it would be interesting to study the glycoprotein distribution of pathological sera.

Thin-layer gel filtration of soluble plant proteins on Bio-Gel P-60 with subsequent PAS and Amido Black staining, has made it possible to separate proteins from polysaccharides[21]. The presence of polysaccharides in protein preparations of plant origin presents a problem in the separation of both macromolecules by different fractionation procedures. Thin-layer gel filtration could in such cases offer a rapid answer about the efficiency of these procedures.

Due to the microgram quantities of material necessary for analysis, thin-layer gel filtration may be anticipated to be particularly useful in cases where only small amounts of material are available. With mammalian cells grown *in vitro*, the amount of material appears to be a limiting factor and an attempt was made, therefore, to separate by thin-layer gel filtration the soluble proteins derived from normal human fibroblasts and HeLa cancer cells. It has been previously shown that HeLa proteins can be separated on Sephadex G-200 columns[5]. A much improved separation can be achieved by thin-layer gel filtration on Sephadex G-200, by which also a comparison with soluble proteins from human fibroblast run simultaneously on the same plate has been made possible (Fig. 5).

The molecular weights determined for the fibroblast and HeLa soluble proteins (Table II) bear a striking similarity to the values obtained on the analysis of soluble rat liver proteins[22]. The molecular weight of the soluble liver proteins has been interpreted in terms of a hypothetical size regularity. The unit of size was assumed to be about 10,000 (u) and 30,000 ($3u$). More than 80% of the soluble liver proteins was demonstrated to belong to size classes whose approximate molecular weights are multiples of these units. The same regularity can be seen in the distribution of the molecular weights of mammalian cells grown *in vitro* (Table II). About 75% of HeLa and fibroblast soluble proteins can be accounted for in terms of the hypothetical size regularity postulated for the soluble liver proteins.

There is an increasing number of papers dealing with the electrophoretic properties of soluble leaf proteins[23–28], but there is only very limited information on their size parameters. This is due partially to technical difficulties in the isolation of soluble leaf proteins which become easily precipitated by some of the components present in plant cells, thus necessitating the use of protective agents[29,30]. Among the better characterized soluble leaf proteins is the so-called "Fraction I protein" which is a major component found in leaf extracts of various plants[29,31–36], and with which a number of enzyme activities are associated[32,33,36]. Column gel filtration has already been successfully applied for the isolation and purification of "Fraction I protein". The molecular weight of "Fraction I protein" is about 600,000 (see ref. 36). Both the high R_M value and the high percentage content of our Fraction I (Fig. 7), amounting to 40% (Table III), found on thin-layer gel filtration of soluble leaf proteins suggest that this fraction is probably identical with the "Fraction I protein" studied by other workers.

The molecular weight for Fraction I on thin-layer gel filtration is about 700,000, a value close to those accepted for "Fraction I protein". In view of the uncertainty in the molecular weight estimations in the range above 240,000 (see ref. 1), this value is to be considered only an approximation. Calculation of the Stokes radius for Fraction I according to eqn. 6 (see ref. 1) gives a value of ~180 Å for the diameter, which is much

higher than that of 120 Å found in electron microscopy[35]. Molecular shrinkage, due to water loss during preparation for electron microscopy, or aggregation of the "Fraction I protein", which has been well established during purification and storage[32], or reversible association–dissociation phenomena could be the explanation for this discrepancy.

In addition to the high molecular weight Fraction I, four other fractions of lower molecular weight in the range from 20 000 to 200 000 could be detected on thin-layer gel filtration of soluble leaf proteins (Fig. 7, Table III). Similar to soluble proteins from mammalian cells grown *in vitro*, three of the fractions show size regularity with a unit size of about 20 000. It is uncertain whether the size classes of 40 000 and 80 000 represent molecules already present in the cell or whether they arise during their isolation as a result of interaction with other cell components. Comparative studies with different isolation procedures, especially those employing various protective agents[37], could help to clarify this question. Thin-layer gel filtration of soluble leaf proteins could prove of value in studies on protein changes associated with leaf aging, as well as with phytopathological processes, since only small amounts of material are needed and single leaves will yield sufficient material for analysis.

One of the potential advantages of thin-layer gel filtration is the possibility of detecting enzymes by using a paper impregnated with the appropriate substrate to give a coloured reaction, or by spraying the paper with the substrate. Enzymes can be specifically located by this means. If no colour reaction is available for detection, an alternative approach is to elute the substance from the paper print with an appropriate buffer, enzyme determinations being then performed with the eluates[38]. As with the column gel technique, the thin-layer method may also be applied to studies of labile enzymes which could be damaged on purification. Besides the small amounts of material required, an additional advantage of the thin-layer method is its greater speed, when compared with the column. Within a few hours after the preparation of the crude enzyme extract, the run can easily be completed. In addition to speed, separation of interfering substances may prove to be another useful property of the method. Preparative thin-layer gel filtration[1] of enzymes can easily provide sufficient material for detecting enzyme activity and also for application of other microanalytical methods, *e.g.* disc electrophoresis or immunodiffusion tests.

The experiments with *o*-diphenol oxidase from mushrooms described in this paper were intended to illustrate some of the potentialities of thin-layer gel filtration of enzymes. There are conflicting data on the molecular weight of this enzyme in a number of papers; most of them agree on a molecular weight of 100 000–130 000 for the purified enzyme[38–41]. The results of thin-layer gel filtration give an estimated molecular weight of 50 000 for the enzyme in the homogenate and of 135 000 for a commercial preparation of "tyrosinase", thus strongly suggesting the presence of enzymatically active subunits that combine under certain conditions to yield a product of higher molecular weight. It is not clear whether this association of smaller units to a component of higher molecular weight is a physiological phenomenon or whether it represents a preparative artifact.

There was a striking difference when homogenates of mushrooms of differing maturity were analyzed. An additional component of higher molecular weight was consistently observed in older mushrooms in which the cap was already open. This need not necessarily reflect the state of the enzyme in the cell, but could also be an

isolation artifact due to interaction of the enzyme with other components appearing in mushrooms of greater maturity.

There was no change in the molecular weight of the 50 000 enzymes, either by precipitation from the homogenate with acetone or by salting out with ammonium sulphate, both of which are treatments routinely employed in the purification of this enzyme. The even greater complexity of the situation is stressed by the presence of two components (35 000 and 50 000) which sometimes could be detected when the homogenate was prepared with the addition of ascorbic acid. Recombination of these subunits would give enzymes with a molecular weight in the range between 100 000–130 000, which would compare well with the molecular weight of purified enzyme preparations. Recombination of subunits could also provide an explanation for the electrophoretic and chromatographic heterogeneity of polyphenol oxidase preparations[42–44].

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REFERENCES

1 B. J. RADOLA, *J. Chromatog.*, 38 (1968) 61.
2 M. GELDMACHER-MALLINCKRODT AND F. MAY, *Z. Physiol. Chem.*, 307 (1957) 179.
3 J. GRONDALE AND F. JACQUELINE, *Rev. Franc. Etudes Clin. Biol.*, 2 (1957) 345.
4 G. CERRIOTTI, *Clin. Chim. Acta*, 2 (1957) 351.
5 B. J. RADOLA, G. KELLNER AND J. FRIMMEL, *Z. Krebsforsch.*, 68 (1966) 42.
6 B. JOHANSSON AND L. RYMO, *Acta Chem. Scand.*, 18 (1964) 217.
7 C. J. O. R. MORRIS, *J. Chromatog.*, 16 (1964) 167.
8 C. H. GRANT AND P. H. EVERALL, *J. Clin. Pathol.*, 18 (1965) 654.
9 K. BERGSTRÖM, *Acta Med. Scand., Suppl.*, 445 (1966) 127.
10 K. BERGSTRÖM AND B. WERNER, *Acta Chir. Scand.*, 131 (1966) 413.
11 P. FLODIN AND J. KILLANDER, *Biochim. Biophys. Acta*, 63 (1962) 403.
12 H. E. SCHULTZE AND J. F. HEREMANS, *Molecular Biology of Human Proteins*, Vol. I, Elsevier, Amsterdam, 1966.
13 G. R. COOPER, in F. W. PUTNAM (Editor), *The Plasma Proteins*, Vol. I, Academic Press, New York, 1961, p. 51.
14 G. SCHUBERT, W. AUERSWALD, W. DOLESCHEL AND H. SCHUBERT, *Klin. Wochschr.*, 43 (1965) 462.
15 L. E. BÖTTIGER, L. A. CARLSON AND S. HEDMAN, *Acta Med. Scand., Suppl.*, 445 (1966) 93.
16 S. D. ROSKES AND T. E. THOMPSON, *Clin. Chim. Acta*, 8 (1963) 489.
17 K. WIRTH, U. ULLMANN, K. BRAND, N. HUTH AND B. HESS, *Klin. Wochschr.*, 43 (1965) 528.
18 G. SCHUBERT, W. DOLESCHEL AND W. AUERSWALD, *Wien. Klin. Wochschr.*, 78 (1966) 629.
19 H. E. MÜLLER, *Med. Klin. (Munich)*, 63 (1968) 125.
20 C. VERGANI AND M. AGOSTONI, *Clin. Chim. Acta*, 16 (1967) 326.
21 B. J. RADOLA AND O. H. K. RICHTER, in preparation.
22 S. SOROF, E. M. YOUNG, R. A. MC BRIDE AND C. B. COFFEY, *Arch. Biochem. Biophys.*, 113 (1966) 83.
23 D. RAMSEN AND M. CALVANICO, *Anal. Biochem.*, 7 (1964) 62.
24 R. C. STAPLES AND M. A. STAHMANN, *Phytopathology*, 54 (1964) 760.

25 F. C. STEWARD, R. F. LYNDON AND J. T. BARBER, *Am. J. Botany*, 52 (1965) 155.
26 C. W. WRIGLEY, H. L. WEBSTER AND J. F. TURNER, *Nature*, 209 (1966) 1133.
27 V. MACKO, G. R. HONOLD AND M. A. STAHMANN, *Phytochemistry*, 6 (1967) 465.
28 B. H. MC CONN, G. E. BECK AND T. C. HALL, *Plant Physiol.*, 43 (1968) 578.
29 N. W. PIRIE, *Ann. Rev. Plant Physiol.*, 10 (1959) 33.
30 M. A. STAHMANN, *Ann. Rev. Plant Physiol.*, 14 (1963) 137.
31 R. W. DORNER, A. HAHN AND S. G. WILDMAN, *Biochim. Biophys. Acta*, 29 (1958) 240.
32 L. MENDIOLÁ AND T. AKAZAWA, *Biochemistry*, 3 (1964) 174.
33 G. VAN NOORT AND S. G. WILDMAN, *Biochim. Biophys. Acta*, 90 (1964) 309.
34 R. AKAZAWA, K. SAIO AND N. SUGIYAMA, *Biochem. Biophys. Res. Commun.*, 20 (1965) 114.
35 T. HASELKORN, H. FERNÁNDEZ-MORÁU AND F. J. HIERAS, *Science*, 150 (1965) 1598.
36 P. W. TROWN, *Biochemistry*, 4 (1965) 908.
37 W. D. LOOMIS AND J. BATTAILE, *Phytochemistry*, 5 (1966) 423.
38 H. DELINCÉE AND B. J. RADOLA, in preparation.
39 D. S. BENDALL AND R. P. F. GREGORY, in J. B. PRIDHAM (Editor), *Enzyme Chemistry of Phenolic Compounds*, Pergamon Press, Oxford, 1963, p. 7.
40 D. KERTESZ AND R. ZITO, *Nature*, 179 (1957) 1017.
41 D. KERTESZ AND R. ZITO, *Biochim. Biophys. Acta*, 96 (1965) 447.
42 J. L. SMITH AND R. C. KRUEGEN, *J. Biol. Chem.*, 237 (1962) 1121.
43 S. BOUCHILLOUX, P. MCMAHILL AND H. S. MASON, *J. Biol. Chem.*, 238 (1963) 1699.
44 R. L. JOLLEY AND H. S. MASON, *J. Biol. Chem.*, 240 (1965) PC 1489.