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ANALYSIS OF GLYCOPROTEIN HORMONES AND OTHER MEDICALLY IMPORTANT PROTEINS BY HIGH-PERFORMANCE GEL FILTRATION CHROMATOGRAPHY

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SUMMARY

Glycoprotein hormones and growth hormone of human origin, and allergen extracts of plant and animal origin have been examined by high-performance liquid chromatography using size exclusion on TSK SW supports. The short times required for elution, compared with low-pressure systems, minimize changes such as aggregation and dissociation. Analysis can be performed with amounts of material too small for examination by conventional gel filtration. The elution behaviour of the glycoproteins is intermediate between those of globular proteins and of dextrans of the same molecular weight. Native luteinizing hormone and follicle stimulating hormone are well separated from their sub-units. Aggregates and sub-units detected in samples of these hormones are not chromatographic artifacts. Differences in the chromatographic profiles of preparations of follicle stimulating hormone are associated in differences in biological and immunological properties. Commercial samples of chorionic gonadotrophin manufactured to official specification contain varying amounts of protein and differ in composition. Analysis on TSK supports confirms that the composition of growth hormone preparations depends on the procedure by which they are obtained, and the main fractions have been examined by electrophoresis. A major peak found in an extract of house dust mite *Dermatophagoides pteronyssinus* is attributed to the P_1 allergen. Other mite species give distinctive elution profiles on chromatography. An extract of pollen from the grass *Dactylis glomerata* was fractionated and the components correlated with proteins in the whole extract separable by electrophoresis. Biological and immunological studies are in progress on the fractions.

INTRODUCTION

Chromatographic supports for separation by molecular size using aqueous media ("gel filtration") were introduced over 20 years ago[1]. Since then, they have been employed extensively for purification and analysis of biological macromolecules such as proteins, polysaccharides and nucleic acids. They permit separation under very mild conditions, suitable for sensitive compounds, and recovery of components

for further investigation. They are, however, unsuitable for use under conditions employed for high-performance liquid chromatography (HPLC) because of the fragility of the gel matrices. Efforts to develop size-exclusion supports suitable for HPLC resulted in the preparation of porous glass beads to which a hydrophilic coat was bonded[2] and, later, other supports which are commercially available[3–6]. The desired characteristics of such supports, approaches to their preparation, and the properties of some of them have been summarized[7]. Much experimental work has been reported using the TSK series of chemically modified silicas (*inter alia* refs. 3 and 7–10) which give efficient separations of a number of proteins without significant adsorption or denaturation. Toste[9] has pointed out that the next stage in evaluation of size-exclusion HPLC is to apply it to biologically important proteins. We now report the use of TSK size-exclusion supports for analysis of preparations of glycoprotein hormones and growth hormone of human origin, and of plant and animal extracts containing allergens. All these materials are medically important but are available in limited amounts, their preparations are usually heterogeneous, and their chemical structure and specific biological activities are not fully characterised.

EXPERIMENTAL

Chromatography

Separations were carried out at ambient temperature (about 20°C) on Toyo Soda TSK G3000SW and G2000SW columns (300 × 7.5 mm), obtained pre-packed. The chromatograph consisted of an Altex 110A pump, Cecil 212A variable wavelength UV detector, Tekman TE 200 recorder operating at 10 mV and Rheodyne 7125 loop injector. 0.1 *M* sodium phosphate buffer pH 7.0 and 0.1 *M* sodium acetate buffer pH 7.0 were used as solvents and a filter was fitted in-line between the solvent reservoir and column. The flow-rate was 0.5 ml/min. Samples up to 100 μl in volume were injected. Fractions (100–500 μl) were collected manually on the basis of the UV traces. The columns were calibrated with protein and dextran standards of known molecular weight in the usual way[9]. Separations on Sephadex G-100 (Pharmacia) were performed on a column, 600 × 10 mm, previously equilibrated with buffer, using 0.1 *M* sodium phosphate buffer pH 7.0 containing 0.02% (w/v) sodium azide, supplied at 0.1 ml/min through an LKB Perpex pump. The Cecil UV detector and Tekman recorder were used.

Electrophoresis

Polyacrylamide gel electrophoresis was used essentially as described by Davis[11] with the spacer and sample gels omitted. The running gels (60 × 5 mm) were stained with 1% (w/v) amido black in 7% (v/v) acetic acid and destained with 7% acetic acid.

Materials

Details of the samples examined are given in Table I. The columns were calibrated with a protein standard kit (Boehringer, Mannheim, G.F.R.), ranging from cytochrome *c* (mol.wt. 12,500) to ferritin (mol.wt. 450,000), and procion-dyed dextran standards (mean mol.wt. 40,000 to 150,000) from Dr. E. A. Johnson (this Institute). All other chemicals were of analytical grade or of the highest grade commercially available.

TABLE I

MATERIALS EXAMINED BY HIGH-PERFORMANCE SIZE-EXCLUSION CHROMATOGRAPHY

Material	*Code/batch number*	*Content per ampoule, or unitage**
Hormones		
FSH	71/270	5.2 μg FSH + 100 μg mannitol
	71/333	4.38 μg FSH + 100 μg mannitol
	76/566	10 μg FSH + 1 mg lactose
	73/519	4.4 μg FSH + 200 μg mannitol
LH	76/569	10 μg LH + 1 mg lactose
	72/20	5 μg LH α sub-unit + 100 μg mannitol
HCG	75/533	2 μg HCG + 100 μg mannitol
	76/508	2 μg HCG α sub-unit + 100 μg mannitol
	75/535	2 μg HCG β sub-unit + 100 μg mannitol
	107	Manufacturer A 2000 units/vial
	0730	Manufacturer B 5000 units/vial
Growth hormone	HWP 40	From acetone preserved glands[12]; approx. 1 unit/mg
	FL 3	From frozen glands[13]; approx. 2.5 units/mg
Allergens		
Dermatophagoides pteronyssinus	77/622	1.19 mg lyophilized mite extract
Acarus siro	77/662	0.96 mg lyophilized mite extract
Dactylis glomerata pollen extract	75/506	6.44 mg lyophilized pollen extract

* Samples obtained within this Institute unless other indicated.

RESULTS AND DISCUSSION

Glycoprotein hormones

Luteinizing hormone (LH) and follicle stimulating hormone (FSH) are glycoprotein hormones produced by the pituitary gland and play a role in sexual development and function. Human chorionic gonadotrophin (HCG) has properties similar to those of LH but is obtained from the urine of pregnant women. All three substances consist of two non-identical protein sub-units to which carbohydrate is attached, and have molecular weights in the range 30,000–40,000. The protein structure of the α sub-unit may be common to all three substances[14]. Methods of isolation are based on chromatographic separation[15]. Purified preparations of the hormones are used for standardizing and calibrating biological and immunological assay systems. HCG is also administered clinically, and its measurement is the basis of diagnostic tests for pregnancy. Because of the small amounts of hormone available, interpretation of bioassays has been complicated by doubts about the homogeneity of reference materials, and the importance of accurate analysis of such preparations is emphasized by problems in radioimmunoassay of LH[16] and by known differences in types of activity[17]. The complete primary structures of the hormones are not yet established because of difficulties in extracting small amounts of hormone from a limited source of supply, in separating the individual substances and the structural heterogeneity of the hormones either through genetic factors or degradation.

A number of potential reference preparations of FSH have been examined using a TSK G3000SW column with the results shown in Fig. 1. These preparations (Table I) are highly purified but differences are apparent in their individual degrees of heterogeneity. The position of the main peak corresponds to that of a globular protein of molecular weight 46,000. Since dextrans of a given weight elute from the column in the position of larger proteins, the hormone behaves as might be predicted for a glycoprotein, assuming a correct molecular weight in the region of 36,000. Peaks eluting after about 20 min are attributable to inert bulking agent in the sample and preservatives. Despite the large amount of carrier, up to 100 times by weight that of the hormone, no interference in the separation is observed. During isolation of FSH, some dissociation into subunits or aggregation may occur. There is chromatographic evidence of aggregated material, in particular, in 71/333 (Fig. 1b) and 76/566 (Fig. 1c), and possibly of sub-units in 76/566 and 73/519 (Fig. 1d). The sample size is of the order of 100 pmoles. Evidence from a variety of biological and immunological assays[18] suggests that the behaviour of 71/720 and 73/519 is similar in such systems but that the other two preparations possess somewhat different properties. Only limited deduction can be made from the chromatographic profiles and it is not possible to make any firm statement about the relationship between composition and activity. It is known, however, that carbohydrate is essential for biological but not for immunological activity. The resolution obtained on the G3000SW column would probably be insufficient to detect whether the carbohydrate is intact or partially degraded.

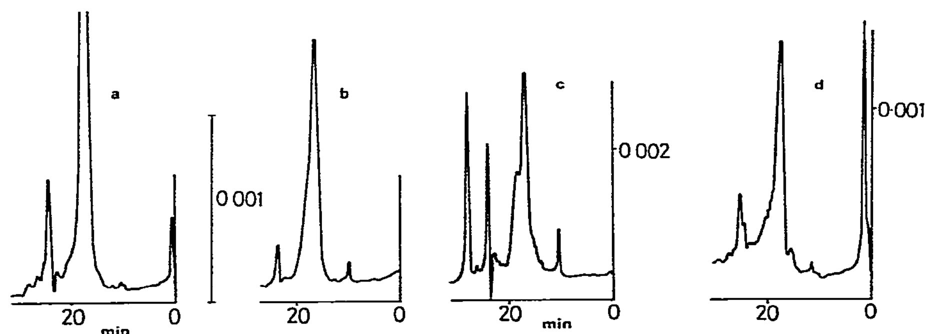

Fig. 1. High-performance size-exclusion chromatography of preparations of FSH from human pituitary glands. Conditions: TSK G3000SW column (300 × 7.5 mm); mobile phase 0.1 *M* sodium acetate buffer pH 7.0; flow-rate 0.5 ml/min; ambient temperature; UV detection at 216 nm. a. Preparation 71/270; b, preparation 71/333; c, preparation 73/519; d, preparation 76/566. For details see Table I. Sample size 5 μg (about 100 pmoles).

Fig. 2 shows the chromatograms obtained with preparations of LH and its α sub-unit. The position of elution of LH is also consistent with its approximate molecular weight. Fig. 2b suggests the presence in the sub-unit preparation of a small amount of material that behaves like the intact hormone on the column. Both preparations appear to contain high-molecular-weight (aggregated) material which is unlikely to be a chromatographic artifact.

Although HCG is available, from urinary sources, in greater amounts than LH or FSH, its isolation presents problems and there is still doubt about the sequence of

Fig. 2. High-performance size-exclusion chromatography of a, LH obtained from human pituitary glands and b, LH α sub-unit. Conditions as Fig. 1. Sample size 5 μg.

Fig. 3. High-performance size-exclusion chromatography of a, HCG from the urine of pregnant women; b, HCG α sub-unit and c, HCG β sub-unit. Conditions as Fig. 1. Sample size 2 μg.

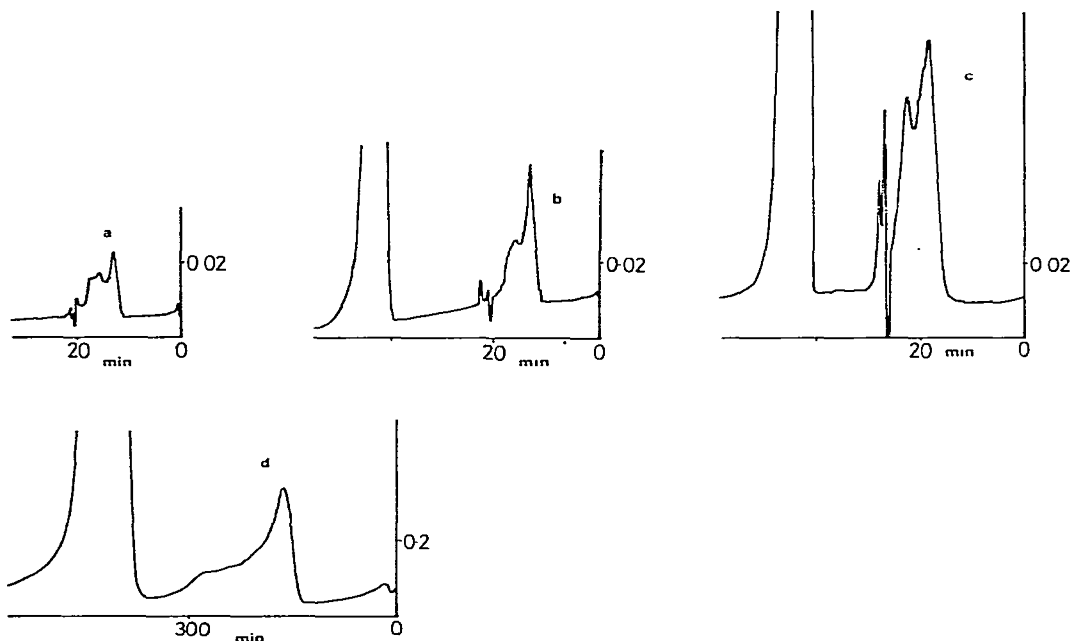

Fig. 4. Chromatography of commercial samples of HCG. a, Manufacturer A. Conditions: TSK G2000SW column (300 × 7.5 mm), mobile phase 0.1 *M* sodium phosphate buffer pH 7.0; flow-rate 0.5 ml/min ambient temperature, UV detection at 280 nm; sample size 400 I.U.; b, as a but manufacturer B; c, as b but 1600 I.U. applied to TSK G3000 SW column; d, manufacturer B. Conditions: Sephadex G-100 column (600 × 10 mm); mobile phase 0.1 *M* sodium phosphate pH 7.0 + 0.02% (w/v) sodium azide; flow-rate 0.1 ml/min, ambient temperature, UV detection at 280 nm, sample size 15000 I.U.

the sub-units[19–21]. The isolated material displays microheterogeneity within the sequence of the α sub-unit. Highly purified preparations of the intact hormone (50 pmoles) and of its two sub-units were chromatographed (Fig. 3). The hormones and the α sub-unit both appear to contain high-molecular-weight material (Figs. 3a and b). Comparison of the traces show that the β sub-unit elutes (Fig. 3c) before the α sub-unit (Fig. 3b) as would be expected since it contains 53 more amino acid residues[21], representing a difference of about 6000 in molecular weight. A mixture of the hormone and both sub-units would be predicted to resolve on the column and this may be the case since peaks are present in the hormone preparation in positions corresponding to those of the two sub-units. The chromatographic behaviour of preparations of HCG manufactured to current pharmaceutical specifications is shown in Fig. 4. Figs. 4a and b are the traces obtained from the two preparations on the G2000SW column. Since highly purified material is not used, the traces are more complex than those in Fig. 3. There is also some difference in composition between the two samples. Loss of resolution on other supports is apparent when Fig. 4b is compared with Fig. 4c, obtained using the same sample but chromatographed on TSK G3000SW, and Fig. 4d where the analysis was carried out on Sephadex G-100.

Growth hormone

Growth hormone is extracted from human pituitaries which are obtained at autopsy and stored either frozen or in acetone until processed. It consists of a single protein chain of 190 amino acid residues and has a molecular weight of 21,500. It can undergo limited degradation without loss of activity and can form aggregates which are biologically inactive. Activity is measured by a number of complex bioassays. Of the various extraction procedures, material obtained by two[12,13] has been administered clinically to stimulate growth of children. These two procedures are known to yield material of somewhat different composition. Figs. 5 and 6 show chromatograms of both types of growth hormone preparation obtained using TSK G2000SW and

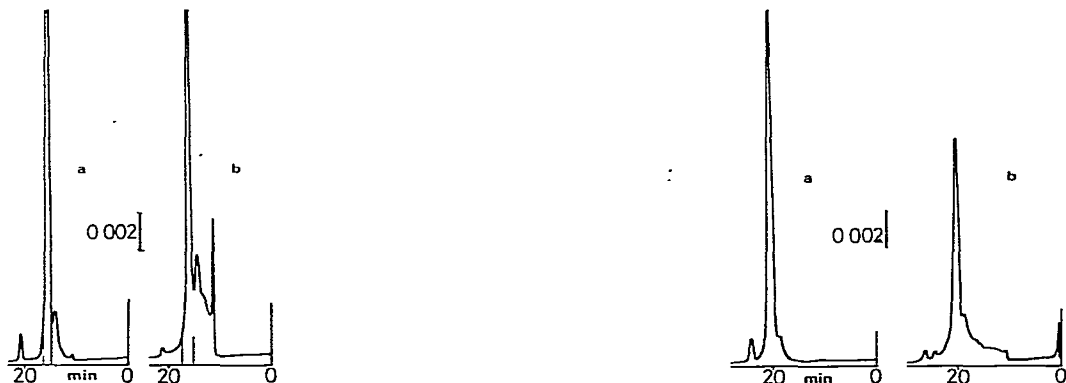

Fig. 5. High-performance size-exclusion chromatography of preparations of human growth hormone. Conditions as Fig. 4a but detection at 210 nm. a, Sample isolated from acetone dried pituitary glands[12]; b, sample isolated from frozen glands[13]. The positions of fractions taken for electrophoretic examination (Fig. 7) are marked. Sample size 10 μg.

Fig. 6. High-performance size-exclusion chromatography of growth hormone. Samples and conditions as Fig. 5a and b, respectively but using TSK G3000SW column.

TSK G3000SW columns, respectively. These figures confirm that the G2000SW column has higher resolving power for substances in this weight range than the G3000SW column. Differences in gross composition are apparent, in particular in the relative amounts of material of larger or smaller size compared with the main peak attributable to growth hormone itself. It is possible that the smaller peak eluting before the main one is due to aggregated material. Comparison of electrophoretic composition of the whole preparations with the main fractions (Fig. 7) shows recovery of hormone without background components although the fractions are not themselves homogeneous, producing one major and one minor band of protein. No evidence of change in composition is obtained when the hormone is rechromatographed so that the other constituents observed in the preparations are not chromatographic artifacts.

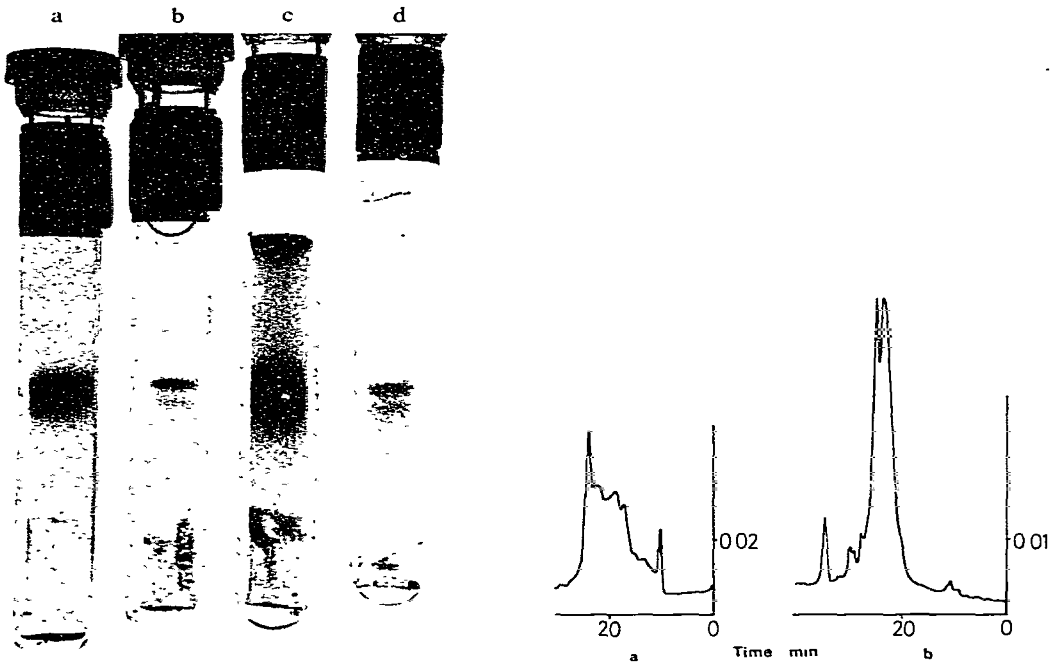

Fig. 7. Polyacrylamide gel electrophoresis of growth hormone preparations and of fractions isolated by chromatography (Fig. 5). Conditions: gels run at pH 8.3 and stained with amido black. Key: a, whole preparation applied in Fig. 5a; b, isolated fraction as marked; c, whole preparation applied in Fig. 5b; d, isolated fraction as marked.

Fig. 8. High-performance size-exclusion chromatography of mite extracts. Conditions as Fig. 5. a, Lyophilized aqueous extract of *Dermatophagoides pteronyssinus* (238 μg); b, lyophilized aqueous extract of *Acarus siro* (96 μg).

Allergens

An allergic response is provoked in man by a variety of natural and artificial substances, termed allergens, which are often protein in character. Although the cause of an allergy can be determined, few allergens themselves have been isolated or fully characterized. However, allergen extracts can be used to desensitize patients, preventing reactions that are often severe and debilitating. We have chromatograph-

ed allergen extracts, prepared from a number of materials, on TSK columns, first to compare their behaviour with their known properties and second, with the aim of recovering purified components for further investigation. Renck and Einarsson[6] have reported the analysis of insect venoms by a similar technique using Shodex OHpak columns under low-pressure conditions.

It is known that the major allergen P_1 in house dust is associated with the presence in the dust of the mite *Dermatophagoides pteronyssinus* and is a glycoprotein of molecular weight about 24,000[22]. Chromatography of a house dust mite extract gave the separation shown in Fig. 8a with a series of peaks. One of these (indicated) elutes at a position expected for a protein of molecular weight about 25,000, as assessed from calibration of the column, but a considerable amount of higher molecular weight material is present. The main component P_1 of the mite fraction F_4P_1 described by Chapman and Platts-Mills[23] elutes in a comparable position. Several other extracts of mite species have been investigated using the TSK columns and patterns characteristic of each have been observed. Fractions have been collected for further investigation by immunological methods and the results will be reported elsewhere. However, the chromatographic profile obtained from *Acarus siro* extract (Fig. 8b) is illustrative of them and can clearly be distinguished from that of *D. pteronyssinus*.

Recent evidence[24] from skin testing suggests that common allergens may be present in certain groups of pollen extracts since many patients cross-react to different species within a group *e.g.* grass pollens. We have applied the size exclusion HPLC procedure to extracts of pollen from *Dactylis glomerata*, a common allergenic grass. Fig. 9a shows the separation obtained and Fig. 10 compares the electrophoretic pattern from the whole extract with those of the fractions collected from the column. Not only are the various components eluted to a considerable extent as discrete peaks on the column, but the electrophoretic examination confirms that there has been no significant loss of any component on the column. These fractions are currently under biological assessment. Unlike a previous method for fractionation[25] of the *D. glomerata* extracts, involving recovery of material after preparative isoelectric focussing, where the presence of extraneous components from the gel could influence subsequent *in vivo* testing, fractions from the TSK column can be examined directly. The improvements in resolution and speed of analysis, and smaller example size associated with use of the TSK column are seen by comparing Fig. 9a with Fig. 9b

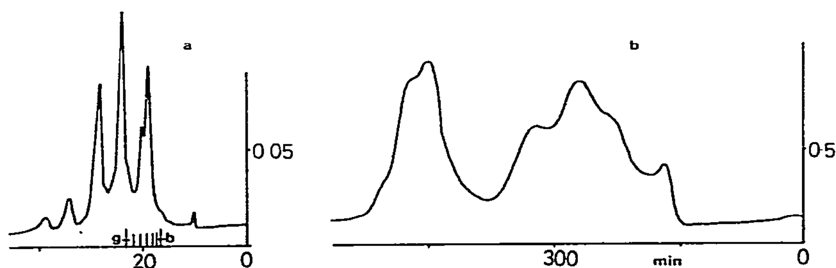

Fig. 9. Chromatography of a pollen extract from the grass *Dactylis glomerata*. a, 300 μg applied to TSK G2000SW. Conditions as in Fig. 4b. Positions of fractions examined in Fig. 10 are indicated; b, 25.7 mg applied to Sephadex G-100. Conditions as in Fig. 4d.

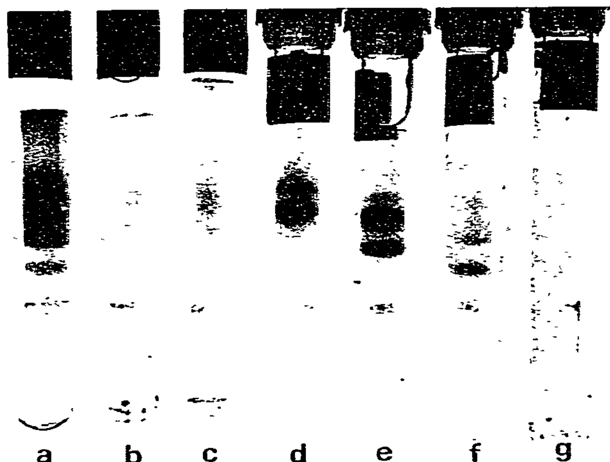

Fig. 10. Polyacrylamide gel electrophoresis of a, pollen extract from *D. glomerata* examined in Fig. 9 and b–g, of fractions isolated from it as shown in Fig. 9. Gels run at pH 4.3 and stained with amido black.

which shows the separation achieved on Sephadex G-100 with the same extract of *D. glomerata*.

CONCLUSION

Results obtained from examination of a variety of protein and glycoprotein preparations by size-exclusion chromatography on TSK columns under HPLC conditions show this to be a powerful addition to the techniques of biochemical analysis. Complex mixtures can be chromatographed rapidly under mild conditions and fractionated material recovered easily for further examination with little or no treatment. The use of simple aqueous media facilitates biological and immunological study of isolated components. Amounts difficult to analyse by previous chromatographic procedures can be studied. The speed of analysis suggest applications to studies on aggregation and dissociation and on binding equilibria. We conclude that this technique should play a valuable role in the identification and study of biologically important molecules.

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