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Note

The use of Sephadex LH-20 to separate dodecyl sulphate and buffer salts from denatured proteins

I. P. GRIFFITH*

Division of Virology, Department of Pathology, University of Cambridge, New Addenbrooke's Hospital, Hills Road, Cambridge CB2 2QQ (Great Britain)

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Chromatography or electrophoresis under denaturing conditions (*e.g.*, in the presence of sodium dodecyl sulphate, urea or guanidine hydrochloride) is increasingly being used to separate complex mixtures of proteins. However, dodecyl sulphate is reported to interfere with the action of trypsin[1], and urea interferes with amino acid analysis. Dodecyl sulphate may be removed by prolonged dialysis[1,2] and urea by gel filtration in 50% acetic acid using Sephadex G-25[3]. However, dialysis requires several days, and the gel filtration is not applicable to proteins insoluble in 50% acetic acid. Anion exchange resins may be used to separate dodecyl sulphate from denatured proteins, but recovery of protein is poor on occasions[4], or requires the presence of 6 *M* urea which then must be removed[5]; moreover, these resins fail to remove the cationic components of buffer salts.

This paper describes a general procedure using Sephadex LH-20 for separating buffer salts (including dodecyl sulphate and non-covalently bound protein stains) from denatured proteins by gel filtration in a volatile solvent.

MATERIALS AND METHODS

Formic acid (AR, 98–100%) was obtained from Fisons (Loughborough, Great Britain) and L-leucine from Roche (Welwyn Garden City, Great Britain). Other reagents (which were, with the exception of β-mercaptoethanol, AR grade) were obtained from British Drug Houses, Poole, Great Britain. Radioisotopes were obtained from the Radiochemical Centre, Amersham, Great Britain. Salt-free cytochrome *c* (horse-heart) was obtained from Boehringer (Mannheim, G.F.R.) and calf serum from Biocult Labs. (Glasgow, Great Britain).

Denaturation of protein

To remove low-molecular-weight material absorbing at 280 nm, calf serum (5 ml) was dialyzed with one change against 500 ml phosphate-buffered saline (Dulbecco's A[11] containing 0.05% NaN_3) for two days, followed by 1000 ml 0.05% NaN_3

* Permanent address to which reprint requests should be sent: Victorian College of Pharmacy, Parkville, Victoria 3052, Australia.

for one day, at 22° after which it was lyophilized. Of this, 20 mg was mixed with 1.5 mg cytochrome *c*, 30 mg sodium dodecyl sulphate, and 2 ml distilled water; an aliquot of sodium dodecyl [^{35}S]sulphate was added, and the protein solution held at 100° for two min. A sample was removed for analysis, and the remainder dialyzed for 24 h at 37° against 5 l of toluene-saturated distilled water containing 0.5 ml β-mercaptoethanol, followed by 500 ml of buffer (3 mg/ml L-leucine and 3 mg/ml Na_2HPO_4, pH 8.3) for 16 h at 37°. To this dialyzed material (after samples were taken for analysis) aliquots of L-leucine-4,5-[^{3}H], [^{32}P]-phosphate and dodecyl [^{35}S]-sulphate were added, together with 5 mg sodium dodecyl sulphate. The mixture was lyophilized and dissolved in 1.5 ml 70% (v/v) formic acid.

Chromatography

Gel filtration was effected at 4° in 70% (v/v) formic acid on a column of Sephadex LH-20 (39 × 1.6 cm) using a solvent flow-rate of 12 ml/h. The column effluent was monitored with an LKB Uvicord operating at 280 nm. Fractions were assayed for absorbance at 394 nm, and for radioactivity.

Radioassay

Samples (0.05 ml) were mixed with 15 ml of scintillant consisting of 3.5 vols. Triton X-100 plus 6.5 vols. of toluene containing 7 g PPO and 0.3 g POPOP per litre. Counts were not corrected for quenching.

RESULTS

It is evident (Table I) that 99% of the dodecyl sulphate was removed by the dialysis procedure described above. However, most of the cytochrome *c* was also lost during the dialysis. Thus, although dodecyl sulphate may be removed by dialysis, the procedure is protracted and may be accompanied by loss of low-molecular-weight proteins.

In contrast, the gel filtration procedure effectively separates both the serum proteins and the cytochrome *c* from the buffer salts (Fig. 1). The protein eluted from

TABLE I

REMOVAL OF DODECYL[^{35}S]SULPHATE FROM DENATURED SERUM AND CYTOCHROME *c* BY DIALYSIS, AND BY GEL FILTRATION ON SEPHADEX LH-20

N.T. = not tested; N.S. = not significant.

Condition	*Optical density*			*Radioactivity (cpm)*
	Wavelength			
	280 nm	*409 nm*	*394 nm*	
Before dialysis	18.2	11.7	N.T.	292,000
After dialysis	17.6	1.5	N.T.	3,200
Recovery	96.7%	12.8%	—	1.1%
Applied to Sephadex column	17.5	N.T.	1.97	198,000
Recovered from Sephadex column (Fractions 13–17 incl., see Fig. 1)	17.3	N.T.	1.985	N.S.
Recovery	99.0%	—	100.8%	0%

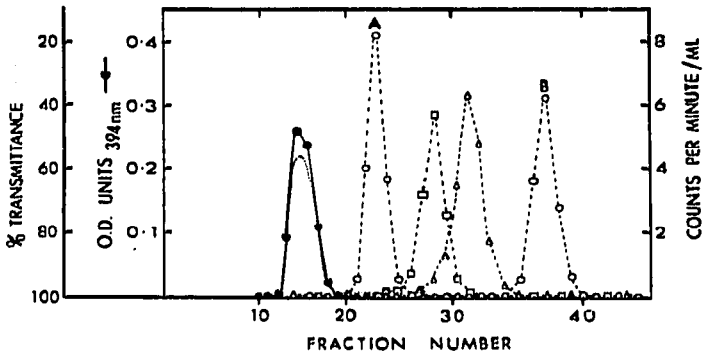

Fig. 1. The use of Sephadex LH-20 to separate protein from buffer salts by chromatography in 70% (v/v) formic acid. The transmission of the column effluent was monitored at 280 nm (···) and the optical density of fractions measured at 394 nm (●—●). Isotopes used were ^{35}S (as sodium dodecyl sulphate, ○---○, c.p.m./ml × $10^{-3\cdot3}$), ^{32}P (as phosphate, □---□, c.p.m./ml × 10^{-3}) and ^{3}H (as L-leucine-4,5-^{3}H, △---△, c.p.m./ml × 10^{-5}).

the column in five fractions (13–17 inclusive) within five hours. None of the phosphate and dodecyl sulphate anions and the leucine zwitterion eluted with these fractions. Recovery of the protein and haem was essentially quantitative as measured by absorption (see Table I), protein recovery on a dry weight basis being 98%. The radioisotope ^{35}S eluted as two peaks. In other experiments free sulphate was found to elute in the position of peak A and [^{14}C]-lauryl alcohol in the position of peak B. As solutions of dodecyl sulphate hydrolyze on storage[6] it is likely that the sodium dodecyl [^{35}S]-sulphate used here was partially hydrolyzed.

It is clear from these results that a complex mixture of denatured proteins may be separated from low-molecular-weight compounds such as leucine, dodecyl sulphate and phosphate. In addition to these compounds, it was possible to separate proteins from glucose, fucose, lauryl alcohol, uridine, UTP, GTP, mixed amino acids, iodide, sulphate, phenol red, calcium, glucosamine and Coomassie Brilliant Blue R250.

DISCUSSION

Anhydrous formic acid is a satisfactory solvent for many proteins[7]. However, attempts to use formic acid alone as the solvent for the gel filtration procedure as described failed, as both phenol red and mixed amino acids eluted over a broad band which encompassed the protein peak; this did not occur when 70% formic acid was used. In addition to improving the solvating power of the formic acid, the presence of water prevents the formic acid freezing at 4°.

In the course of many hours at room temperature, anhydrous formic acid will formylate proteins[8]. However, proteins are routinely exposed for hours to 70% aqueous formic acid at room temperature during the cyanogen bromide cleavage procedure[9] and to formic acid buffer at pH 1.9 during peptide mapping[2], and exposure of proteins to 70% formic acid for a few hours at 4° is unlikely to alter them chemically.

Proteins may be recovered from the column effluent by precipitating them with diethyl ether or by removing the solvent by lyophilization. However, proteins recovered in these ways, although readily soluble in 6 *N* HCl, are generally insoluble

in aqueous solution near neutrality and are thus digested with difficulty by trypsin. This problem may be overcome by removing the formic acid by dialysis against water followed by 0.5% ammonium bicarbonate. This frequently leaves proteins as a fine suspension which is rapidly digested by trypsin[10].

Protein may be eluted from stained acrylamide gels stored dry by soaking the latter in 70% formic acid. This protein is available for further analysis after removal of stain by the procedure described in this work. Although not tested, it is probable that other small molecules, such as urea, guanidine hydrochloride and iodoacetamide may be separated from denatured protein by the method described here, which seems to have the advantage over other desalting procedures in being quite rapid, and of general application.

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