A Simple Sensitive Method, Applicable to Small Polypeptides, for the Determination of Proteins in Polyacrylamide Gels after Isoelectric Focusing

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(Received 15 August 1977)

A simple method is described for the determination of polypeptides and proteins in polyacrylamide gels after isoelectric focusing. Precipitates formed in trichloroacetic acid, under controlled conditions, are quantified densitometrically by measuring the proportion of light scattered. The procedure is of particular value in its applicability to smaller polypeptides, with mol.wts. of 3000–6000, which are not fixed adequately in gels by other procedures currently in use. The working range, over which polypeptide concentration is proportional to the effective absorbance, is approx. 1–30 μg per component.

Protein components separated by polyacrylamide-gel electrophoresis have been most usually quantified by densitometry of gels stained variously with dyes such as Amido Black 10B (Kruski & Narayan, 1968; McEwen, 1968), Fast Green (Gorovsky *et al.*, 1970), Coomassie Brilliant Blue R250 (Fishbein, 1972) and the vinyl sulphone derivative of Remazole Brilliant Blue R (Datyner & Finnimore, 1973). Coomassie Blue, the most sensitive of these stains (Fazekas de St. Groth *et al.*, 1963), is used most commonly. Absorbance measurements of the dye eluted from such stained gels have also been used (Johns, 1967), since this method has the advantage of extending the range of protein concentrations over which Beer's law is obeyed, and avoids the effects of bandwidth on this relationship (Fenner *et al.*, 1975). U.v.-spectrophotometric scanning of unstained gels (Dravid *et al.*, 1969) is less sensitive than the other two methods (Watkin & Miller, 1970).

The measurement of protein components separated by isoelectric focusing in polyacrylamide gels is complicated by the presence of the carrier ampholytes, which share various of the physicochemical properties of proteins. Thus scanning of unstained gels at 280 nm is inapplicable, owing to the variable background absorbance contributed by the separated ampholytes (Righetti & Drysdale, 1971). The staining methods used after electrophoresis usually produce insoluble ampholyte-dye complexes. This has been avoided by staining in methanolic or ethanolic solutions (Riley & Coleman, 1968; Awdeh, 1969; Spencer & King, 1971; Vesterberg, 1971), conditions where the ampholyte-dye complexes are relatively more soluble than protein-dye complexes. Alternatively the gel has been washed extensively in trichloroacetic acid, to fix the protein and remove carrier ampholyte, before staining as after electro-

phoresis (Storring *et al.*, 1972). These methods are generally tedious and time-consuming and/or less sensitive than those applied after polyacrylamide-gel electrophoresis.

Additional problems are encountered after electrofocusing or electrophoresis (especially under alkaline conditions) of smaller polypeptides, since these are difficult to fix even in highly cross-linked gels. Thus after isoelectric focusing of corticotropin and corticotropin-(1–24)-tetracosapeptide in polyacrylamide gel, direct staining methods (Awdeh, 1969; Spencer & King, 1971; Vesterberg, 1971) proved very insensitive, because these polypeptides, and probably also their dye complexes, are soluble in methanolic or ethanolic solutions; neither did trichloroacetic acid (10%, w/v) fix these polypeptides efficiently enough to permit elution of ampholyte before staining in aqueous solvents.

At trichloroacetic acid concentrations higher than are normally used, such polypeptides are precipitated quantitatively, and light-scattering measurements of the precipitates gave a sensitive method for their estimation.

Materials and Methods

Polypeptides and proteins

Ampoules of freeze-dried preparations contained (approx. values): pig corticotropin (50 μg) with 5 mg of lactose (coded 59/17); corticotropin-(1–24)-tetracosapeptide (100 μg) with 15 mg of mannitol (coded 73/570); oxytocin (20 μg ; approx. 50% as dimer) with 5 mg of lactose; [lysine]vasopressin (20 μg ; approx. 50% as dimer) with 5 mg of lactose; pig glucagon (1 mg) with 100 mg of lactose (Novo Industri A/S, Copenhagen, Denmark). These ampoules were assumed to contain the nominal quantities, except for

corticotropin and corticotropin-(1-24)-tetracosapeptide, where isoelectric focusing had indicated that these polypeptides (the main components) represented 70% (w/w) and 85% (w/w) respectively of the nominal contents. The sources of the following proteins were as indicated: pig gonadoliberein (synthetic; Abbott Laboratories, Chicago, IL, U.S.A.); pig insulin (Wellcome Reagents, Beckenham, Kent, U.K.); salmon calcitonin (synthetic; Armour Pharmaceutical Co., Eastbourne, Sussex, U.K.); bovine serum albumin [crystalline; Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K.].

Isoelectric focusing in polyacrylamide gel

Gels were prepared at room temperature (20°C). Stock solutions of ampholyte (40%, w/v, pH 3.5-10; LKB Produkter AB, Bromma, Sweden), acrylamide (40%, w/v; Eastman Kodak Co., Rochester, NY, U.S.A.), *NN'*-methylenebisacrylamide (0.8%, w/v; Eastman Kodak Co.) and riboflavin (0.002%, w/v; BDH Chemicals, Poole, Dorset, U.K.) were mixed with freshly prepared solutions of dithiothreitol (4mm; SLR; Fisons Scientific, Loughborough, Leics., U.K.) and sucrose (60%, w/v; Aristar, BDH Chemicals) to give a gel solution containing ampholyte (1%, w/v), acrylamide (15%, w/v), *NN'*-methylenebisacrylamide (0.3%, w/v), sucrose (15%, w/v), riboflavin (0.0001%, w/v) and dithiothreitol (1mm). Gels for isoelectric focusing of serum albumin were prepared from a solution containing 7.5% (w/v) acrylamide and 0.15% (w/v) *NN'*-methylenebisacrylamide. After degassing the gel solution, gels were cast in Pyrex tubes (0.5cm × 8cm); the well of the Suba-Seal (Gallenkamp, London E.C.2, U.K.), which stoppered one end of the tube, had first been filled with 60% (w/v) sucrose to ensure a smooth lower gel surface. Polymerization was carried out for 1h in front of a daylight fluorescent lamp (Shandon Southern Products, Runcorn, Cheshire, U.K.), and the gels were then equilibrated at 4°C for 2h.

The samples were dissolved in 10mm-HCl/0.5% (w/v) mercaptoethanol, diluted 1:1 with sucrose (40%, w/v) and introduced on to the gel surface in 100μl. The electrofocusing of serum albumin, insulin, oxytocin and [lysine]vasopressin was carried out in the absence of the reducing agents dithiothreitol and mercaptoethanol in the gel solution and sample diluents. Isoelectric focusing was undertaken at 4°C, in the same apparatus as used for electrophoresis (Davis, 1964) with the anode uppermost; the anolyte was phosphoric acid (10mm) and the catholyte NaOH (20mm), and a constant voltage of 340V for 1h was used. A short period of isoelectric focusing was used (too short for the development of an optimal pH gradient), since analytical separations only were required, and some of the polypeptides

investigated, e.g. corticotropin-(1-24)-tetracosapeptide and [lysine]vasopressin, were very basic, with isoelectric points of 11 or more. Highly cross-linked gels were chosen to facilitate fixing of the smaller polypeptides studied.

Trichloroacetic acid precipitation

The gels, removed from their tubes immediately after isoelectric focusing, were immersed in 16ml of trichloroacetic acid; the final acid concentration, after equilibration, was therefore approx. 90% (w/v) of that added. Gels, kept at 4°C, were first equilibrated at room temperature (20°C) for about 1h before being scanned for light scattering.

Light-scattering measurements

The gels (in silica tubes of 8mm internal diam.) were scanned at 350 or 450nm in a densitometer (SP.1809; Pye-Unicam, Cambridge, U.K.), with the use of a slit unit of 0.1mm and a longitudinal sample mask of 1.5mm. The densitometer was fitted to an SP.1800 spectrophotometer (Pye-Unicam), for which the slit width was set at 1mm and the reference beam attenuated with a 1.2A gauze, and a linear recorder (AR 55; Pye-Unicam). The shortest wavelength that could be used without excessive baseline absorbance in ampholyte-containing gels was 350nm; a wavelength of 450nm, used for some experiments, decreased the sensitivity of measurements to 75% of that at 350nm, but the results were otherwise very comparable. The proportion of light scattered was measured as the effective absorbance [$\log(I_0/I)$], and the area under the chart peak (in arbitrary units, which varied between experiments with the wavelength at which the gels were scanned and the sensitivity setting used for the densitometer) was estimated with a fixed-index Allbrit polar planimeter (W. F. Stanley and Co., London S.E.9, U.K.).

Results and Discussion

Optimum conditions for trichloroacetic acid precipitation

Trichloroacetic acid concentration. Trichloroacetic acid (30%, w/v) produced the densest precipitate with corticotropin-(1-24)-tetracosapeptide and calcitonin and, to a less pronounced degree, with corticotropin, and was therefore used in all subsequent work; 25% (w/v) trichloroacetic acid and lower concentrations, or 40% (w/v) and higher concentrations, gave progressively less precipitation (Fig. 1). The precipitation of serum albumin was little affected by the trichloroacetic acid concentration used over the range examined.

Temperature. Precipitation was undertaken at 4°C rather than at 20°C, since this increased the amount of precipitate [about 2-fold for corticotropin-(1-24)-

Fig. 1. Effects of trichloroacetic acid concentration on precipitation after isoelectric focusing

●, Corticotropin-(1-24)-tetracosapeptide (17 μ g); ○, calcitonin (20 μ g); ■, corticotropin (16 μ g); ▲, serum albumin (20 μ g). Gels were scanned at 350 nm.

tetracosapeptide], probably by decreasing the diffusion rate of the smaller polypeptides, as well as the solubility of the trichloroacetic acid salt formed.

Time. Precipitation took several hours for completion, as judged by the extension of a disc of uniform density through the whole cross-section of the gel, and therefore an overnight period (16 h) was allowed for this.

Ampholyte precipitation. Trichloroacetic acid concentrations of 30% (w/v) and higher (up to 40% w/v) produced considerable ampholyte precipitation in gels when added at 4°C, but not at 20°C; at a concentration of 45% (w/v) acid the degree of ampholyte precipitation was less than at 40% (w/v). For gels prepared with 1% (w/v) ampholyte, the ampholyte precipitation could be completely reversed by equilibrating the gels at 20°C for 1 h, thereby making them suitable for the densitometric scanning of peptide components. Acid-ampholyte precipitates in gels containing 2% (w/v) ampholyte did not disappear entirely at 20°C, probably owing to the small proportion of higher-molecular-weight species present in the carrier ampholyte (Righetti *et al.*, 1975).

Stability of precipitate. This was much greater at 4°C than at 20°C. For corticotropin-(1-24)-tetracosapeptide, the density of the precipitate present after 1 week, with gels kept at 4°C, was approx. 80% of that found initially, but with gels kept at 20°C was less than 30% of the initial value. Precipitates formed with polypeptides larger than corticotropin-(1-24)-tetracosapeptide, namely calcitonin, glucagon and corticotropin, showed no significant loss in their density after 1 week at 4°C.

Applicability of the precipitation procedure to different polypeptides

Polypeptides larger than corticotropin-(1-24)-tetracosapeptide (mol.wt. approx. 3000), namely calcitonin, glucagon, corticotropin and insulin, with mol.wts. of 3435, 3483, 4567 and 5764 respectively, were precipitated readily in 30% (w/v) trichloroacetic acid after isoelectric focusing. Polypeptides with molecular weights of less than 3000 either gave no detectable precipitates {[lysine]vasopressin (mol.wt. 1024) and oxytocin (mol.wt. 1007) and their respective dimers} or, for gonadoliberein (mol.wt. 1182), precipitates at 4°C, which disappeared when the gels were equilibrated at 20°C.

Quantification of trichloroacetic acid precipitates

The effective absorbance measurements are proportional to the quantity of peptide precipitated (Fig. 2). This relationship is linear within the ranges approx. 1–30 μ g per peptide component. In a number of instances the standard curve fails to extrapolate through the origin; this shows itself as a small displacement after isoelectric focusing of the smaller polypeptides corticotropin-(1-24)-tetracosapeptide and calcitonin, and suggests some diffusion before precipitation; once formed, the precipitates themselves appear to remain fixed (see above). The sensitivity of the method, after isoelectric focusing, is about 1–2 μ g per peptide component, and the agreement between replicates is good.

The slopes of the linear portions of the standard curve vary with different polypeptides or proteins (Fig. 2). This may be related to the number of free amino groups per unit mass or mol, especially for the smaller polypeptides, where the number of trichloroacetic acid molecules complexed would modify considerably the effective mass precipitated. Thus corticotropin-(1-24)-tetracosapeptide, which contains eight of the nine free amino groups of corticotropin, also shows a steeper slope than the latter on a mass basis, though not on a molar basis (Fig. 2b). Other factors could also influence the density of the precipitate and so the proportion of light scattered. For glucagon the concentration-dependent aggregation, which occurs under acid conditions (Beavan *et al.*, 1969), might account for the inflexion in the slope observed at the highest dose (Fig. 2d). However, the minor components of corticotropin-(1-24)-tetracosapeptide [a more acidic component, representing a form of the molecule with the methionine residue oxidized (Dixon, 1955)] and of corticotropin [a more anodal (deamidated) and a more cathodal component] gave standard curves with slopes similar to those of their respective main components. As for most other methods for quantifying proteins, accurate determinations require

Fig. 2. Relationship between amount of peptides electro-focused and density of precipitate

(a) Corticotropin-(1-24)-tetracosapeptide (means $\pm$ s.e.m. for four determinations); (b) corticotropin-(1-24)-tetracosapeptide (●) and corticotropin (■) (means for duplicate or triplicate determinations); (c) calcitonin (○) and corticotropin (■); (d) corticotropin-(1-24)-tetracosapeptide (●), glucagon (□) and insulin (Δ) (means of duplicate determinations). Gels in (a), (b) and (d) were scanned at 450 nm, and gels in (c) at 350 nm.

that a standard is used of the same material as that being measured.

Conclusions

Although trichloroacetic acid precipitation has been used to fix and locate protein components in polyacrylamide gels, particularly after isoelectric focusing (Finlayson & Chrambach, 1971), it has not yet been used to quantify such components.

The advantages of the method reported here lie in its simplicity (requiring no destaining procedures) compared with other procedures currently in use, and particularly in its applicability to smaller polypeptides. The precipitates formed in this way appear, in most cases, to be as stable as protein-stain complexes (Davis, 1964; Chrambach *et al.*, 1967), and the working range of approx. 1–30 μ g per component, is similar to that of other densitometric methods (Fenner *et al.*, 1975).

The method was also applicable after electrophoresis in polyacrylamide gel.

We are very grateful to Dr. E. A. Johnson for valuable advice and criticism.

References

- Awdeh, Z. L. (1969) *Sci. Tools* **16**, 42–43
- Beaven, G. H., Gratzner, W. B. & Davies, H. G. (1969) *Eur. J. Biochem.* **11**, 37–42
- Chrambach, A., Reisfeld, R. A., Wyckoff, M. & Zaccari, J. (1967) *Anal. Biochem.* **20**, 150–154
- Datnyer, A. & Finnimore, E. (1973) *Anal. Biochem.* **52**, 45–55
- Davis, B. J. (1964) *Ann. N. Y. Acad. Sci.* **121**, 404–427
- Dixon, H. B. F. (1955) *Biochim. Biophys. Acta* **18**, 599–600
- Dravid, A. R., Fredén, H. & Larsson, S. (1969) *J. Chromatogr.* **41**, 53–60
- Fazekas de St. Groth, S., Webster, R. G. & Datnyer, A. (1963) *Biochim. Biophys. Acta* **71**, 377–391
- Fenner, C., Traut, R. R., Mason, D. T. & Wikman-Coffelt, J. (1975) *Anal. Biochem.* **63**, 595–602
- Finlayson, G. R. & Chrambach, A. (1971) *Anal. Biochem.* **40**, 292–311
- Fishbein, W. N. (1972) *Anal. Biochem.* **46**, 388–401
- Gorovsky, M. A., Carlson, K. & Rosenbaum, J. L. (1970) *Anal. Biochem.* **35**, 359–370
- Johns, E. W. (1967) *Biochem. J.* **104**, 78–82
- Kruski, A. W. & Narayan, K. A. (1968) *Biochim. Biophys. Acta* **168**, 570–572
- McEwen, B. S. (1968) *Anal. Biochem.* **25**, 172–180
- Righetti, P. & Drysdale, J. W. (1971) *Biochim. Biophys. Acta* **236**, 17–28
- Righetti, P., Righetti, A. B. B. & Galante, E. (1975) *Anal. Biochem.* **63**, 423–432
- Riley, R. F. & Coleman, M. K. (1968) *J. Lab. Clin. Med.* **72**, 714–720
- Spencer, E. M. & King, T. P. (1971) *J. Biol. Chem.* **246**, 201–208
- Storring, P. L., Burns, T. W., Furnival, B. E., Hales, C. N., Langley, P. & Hartree, A. S. (1972) *J. Endocrinol.* **55**, 543–554
- Vesterberg, O. (1971) *Biochim. Biophys. Acta* **243**, 345–348
- Watkin, J. E. & Miller, R. A. (1970) *Anal. Biochem.* **34**, 424–435