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Polymerization of Proteins with Glutaraldehyde

SOLUBLE MOLECULAR-WEIGHT MARKERS

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Glutaraldehyde is well known for its ability to react with proteins and to produce insoluble cross-linked aggregates. In contrast with this situation, conditions are described here which yield covalently linked soluble protein oligomers. The procedure is applicable to a wide range of proteins, and by slight variation in the reaction conditions, soluble polymers in the molecular weight range 3×10^4–2×10^7 were produced. The products are valuable as molecular-weight markers, e.g. in sodium dodecyl sulphate–polyacrylamide-gel electrophoresis. The inherent similarities of these oligomers make them superior to commercial molecular-weight protein markers, which may have marked differences in composition and charge.

As part of a collaborative project aimed at relating the effect of molecular size on immunogenicity of proteins, we have recently been investigating procedures designed to produce high-molecular-weight protein aggregates via intermolecular cross-bridges, and to this end we have studied the reaction of glutaraldehyde with various proteins. Many other bifunctional reagents for cross-linking proteins have been described (Fasold *et al.*, 1971), but in general, they have been used to produce intramolecular rather than intermolecular bridges. The literature on the reaction of glutaraldehyde with biological systems is extensive. Hopwood (1967, 1969*a*) has reviewed its application in fixation. Many authors have described its use for the preparation of insoluble protein aggregates (Hopwood, 1969*b*; Hopwood *et al.*, 1970; Avrameas & Ternynck, 1969), and insoluble derivatives of certain enzymes, e.g. papain (Jansen & Olson, 1969; Ottesen & Svensson, 1971), carboxypeptidase (Quiocho & Richards, 1964, 1966), subtilisin (Ogata *et al.*, 1968), trypsin (Habeeb, 1967) and catalase (Schejter & Bar-Eli, 1970); in many cases substantial enzymic activity was preserved. It has also been used for the conjugation of proteins and enzymes (Ottesen & Svensson, 1971; Avrameas, 1969) and for coupling proteins to various matrices (Avrameas *et al.*, 1969; Weston & Avrameas, 1971). In certain instances soluble protein derivatives have been described (Hopwood, 1969*b*; Ottesen & Svensson, 1971; Habeeb & Hiramoto, 1968; Griffith, 1972). In most of the studies, rather high glutaraldehyde concentrations have been employed, a procedure that is likely to produce extensive derivative formation but not necessarily extensive cross-linking. To preserve native protein conformation, and to ensure the solubility of the resultant polymers by retention of native hydrophilic amino acid residues, we sought to produce cross-linked polymers with minimum amino acid substitution: to this end, we have used low glutaraldehyde concentrations, together with high protein concentrations to facilitate intermolecular reaction. In characterizing certain of the products on sodium dodecyl sulphate–polyacrylamide-gel electrophoresis, we were impressed with their possible application as molecular-weight markers. In the present paper these glutaraldehyde oligomers are compared with the proteins commonly used as molecular-weight markers.

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Materials and Methods

Materials

Glutaraldehyde (25%) was obtained from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K., and was used without purification. Bovine serum albumin (crystalline) and lysozyme were purchased from BDH Ltd., Poole, Dorset, U.K. Cytochrome *c*, bovine haemoglobin (type 1, twice crystallized) and ovalbumin were obtained from Sigma (London) Chemical Co. Ltd., London, S.W.6, U.K. Asparaginase from *Erwinia carotovora* was a gift from Dr. H. E. Wade, Microbiological Research Establishment, Porton. Acrylamide, methylenebisacrylamide and *NNN'N'*-tetramethylenediamine were obtained from Kodak Ltd., Kirkby, Liverpool, U.K. Ammonium persulphate, trichloroacetic acid, sodium dodecyl sulphate (sodium lauryl sulphate, specially pure) and Coomassie Brilliant Blue R-250 were purchased from BDH. Dextran Blue and Sepharose

4B were obtained from Pharmacia (Great Britain) Ltd., 75 Uxbridge Road, London W5 5SS, U.K.

Amino acid analyses

Protein samples (1.5mg) were hydrolysed with 6M-HCl in sealed tubes under N_2 for 22h at 115°C, and the hydrolysates were examined on a Technicon Auto-Analyzer.

Protein concentrations and asparaginase activity

These were determined by automated colorimetric procedures (Wade & Phillips, 1971).

Sodium dodecyl sulphate–polyacrylamide-gel electrophoresis

For this, 5% (w/v) acrylamide gels (7.0cm × 0.5cm) were prepared as described by Weber & Osborn (1969), except that the amount of cross-linker was decreased by one-third. Proteins were incubated at room temperature overnight in 0.1M-sodium phosphate, pH7.1, containing sodium dodecyl sulphate (1%, w/v) and β-mercaptoethanol (0.5%). Sample preparation varied with the protein mixture under study, but for a typical run 10μl of protein (10mg/ml), 10μl of sucrose (50%, w/v) and 5μl of gel buffer (sodium phosphate, 0.075M, pH7.1, containing sodium dodecyl sulphate 0.1%, w/v) were mixed together and samples (10μl) added to each gel. Identical resolution was observed if proteins were incubated in gel buffer in place of the above 1% sodium dodecyl sulphate buffer solution. Samples were run at 5–8mA/gel for 3–4h. Specific conditions are indicated in the text. Proteins were precipitated and bands were stained with a solution of 0.05% Coomassie Brilliant Blue in water–methanol–trichloroacetic acid (5:5:1, v/v/w). The gels were destained by soaking in water–methanol–trichloroacetic acid (38:2:3, v/v/w), and the clear gels were stored in 7.5% (v/v) acetic acid.

The protein patterns were recorded by scanning the gels with a Joyce–Loebl Chromoscan apparatus, and protein mobilities were measured from the densitometer tracings. The gels shrink and swell during staining and destaining and were therefore equilibrated in 7.5% acetic acid before scanning. Electrophoretic mobility (cm) was measured from the top of the gel.

Results

Influence of protein concentration on degree of polymerization

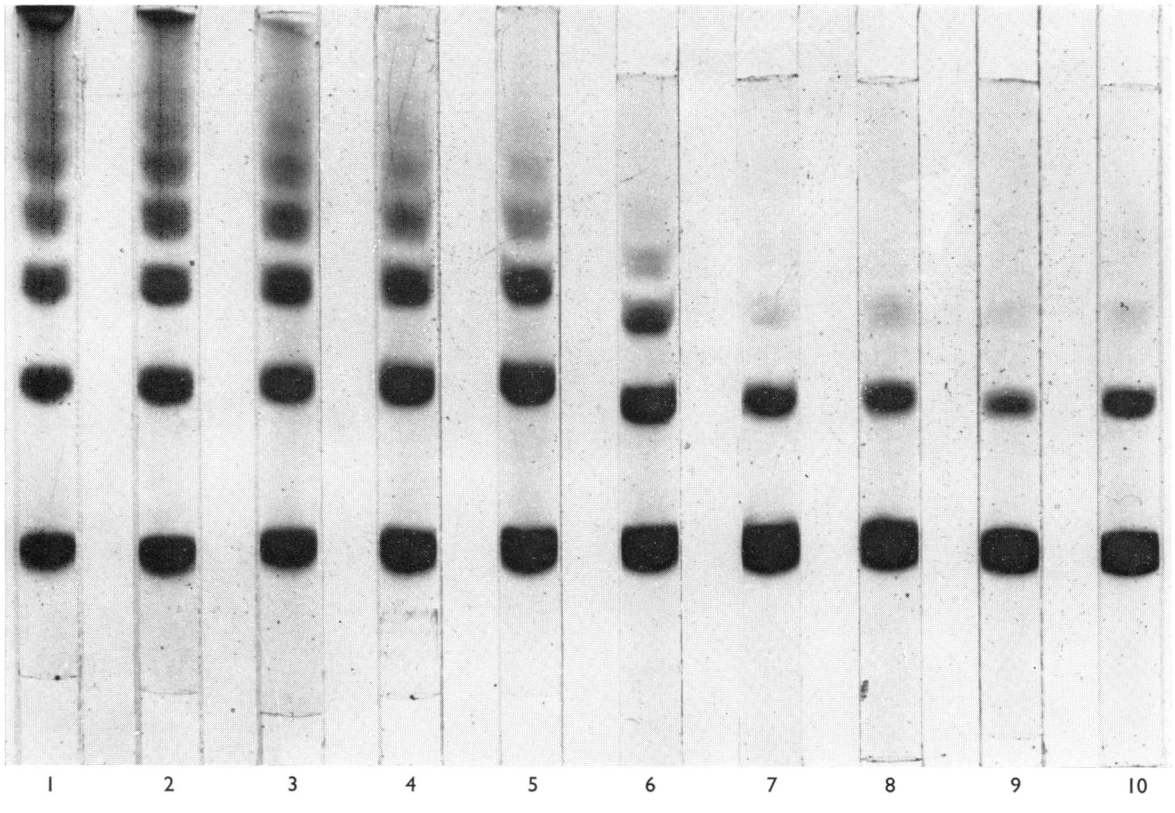
Equal volumes of albumin (samples 1–9, Plate 1) at various concentrations (6.25–175mg/ml) were treated with constant proportions (by weight) of glutaraldehyde as described in the explanation to Plate 1. The albumin solutions were mixed on a vortex mixer while the glutaraldehyde was added. Mixing was continued for 1min and the solutions were incubated at room temperature for 24h. All but sample 9 turned yellow within 10min of addition of glutaraldehyde. The products were examined by sodium dodecyl sulphate–polyacrylamide-gel electrophoresis and the resultant gels are shown in Plate 1. Similar protein patterns were obtained after the samples had been stored at 4°C for 6 weeks. From the relative mobilities of the bands it is apparent (see below) that the bands represent monomer, dimer, trimer, etc. from bottom to top.

Influence of glutaraldehyde concentration on degree of polymerization

This study was carried out with albumin, asparaginase, lysozyme and haemoglobin at protein concentrations of 50–200mg/ml. The products were examined electrophoretically, and typical examples for lysozyme and haemoglobin are illustrated in Plates 2(*a*) and 2(*b*) respectively. All samples of lysozyme turned yellow and with 10% glutaraldehyde a viscous suspension was produced but this readily dissolved on dilution. Haemoglobin behaved identically to lysozyme. Products obtained by treating albumin samples (250μl, 200mg/ml) with 10μl amounts of glutaraldehyde (0.05%–5%) were all soluble, and polymers up to the decamer were resolved electrophoretically. Treatment of asparaginase (60μl, 125mg/ml) with 2μl amounts of glutaraldehyde (0.1%–1%) gave soluble polymers with no loss of enzymic activity, but similar treatment with 2.5% glutaraldehyde yielded a precipitate.

Oligomeric nature of the treated proteins

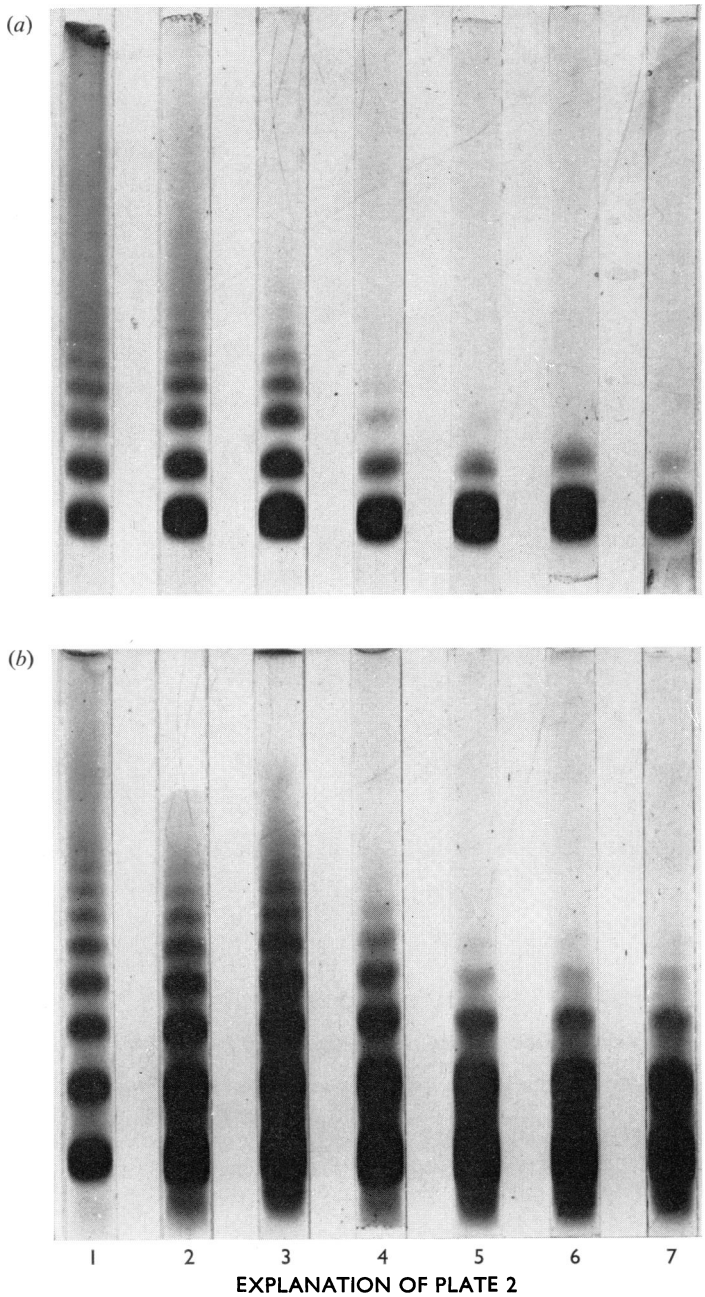
Although the appearance of the protein bands in the gels indicated that discrete species were produced, their homologous nature was revealed when electrophoretic mobilities were measured from densitometer tracings of the gels. The inverse relationship between mobility and logarithm of molecular weight was first suggested by Shapiro *et al.* (1967) and has been confirmed by others (Weber & Osborn, 1969; Dunker & Rueckert, 1969). Scans of polymerized asparaginase, albumin, haemoglobin and lysozyme are shown in Fig. 1, together with plots of logarithm of molecular weight (estimated from the degree of polymerization) of the protein oligomers against their mobilities. In the presence of sodium dodecyl sulphate, haemoglobin is dissociated into subunits and the observed haemoglobin polymers are cross-linked multiples of these subunits.



EXPLANATION OF PLATE 1

Influence of protein concentration on the degree of polymerization of bovine serum albumin by glutaraldehyde

Albumin (200mg/ml) in 0.1M-sodium phosphate, pH7.5, was diluted in the same buffer to give samples 1–9 containing 175, 150, 125, 100, 75, 50, 25, 12.5 and 6.25mg/ml respectively. To samples of 1–9 (200μl) was added 7, 6, 5, 4, 3, 2, 1, 0.5 and 0.25μl respectively of 5% glutaraldehyde. Sample 10 is an untreated control. Reaction procedure is described in the text. Products were diluted to 10mg/ml (sample 9 used directly) and samples (30μg) prepared as described in the Materials and Methods section were run at 8mA/gel for 200min.



EXPLANATION OF PLATE 2

Protein polymerization as a function of glutaraldehyde concentration

(*a*) Samples (200μl) of lysozyme (50mg/ml) in 0.1 M-sodium phosphate buffer, pH 7.5, were mixed for 30s with 2μl amounts of 10, 5, 2.5, 1, 0.5, 0.1 and 0.05% glutaraldehyde then left for 48h at 20°C to give products 1–7 respectively. These were finally diluted 3.5-fold and samples (45μg) were run at 5mA/gel for 3.5h. (*b*) Samples (50μl) of haemoglobin (200mg/ml) were treated as in (*a*) with 2μl amounts of 10, 5, 2.5, 1, 0.5, 0.1 and 0.05% glutaraldehyde to give products 1–7 respectively. These were diluted 20-fold and samples (30μg) run at 5mA/gel for 3.5h.

J. W. PAYNE

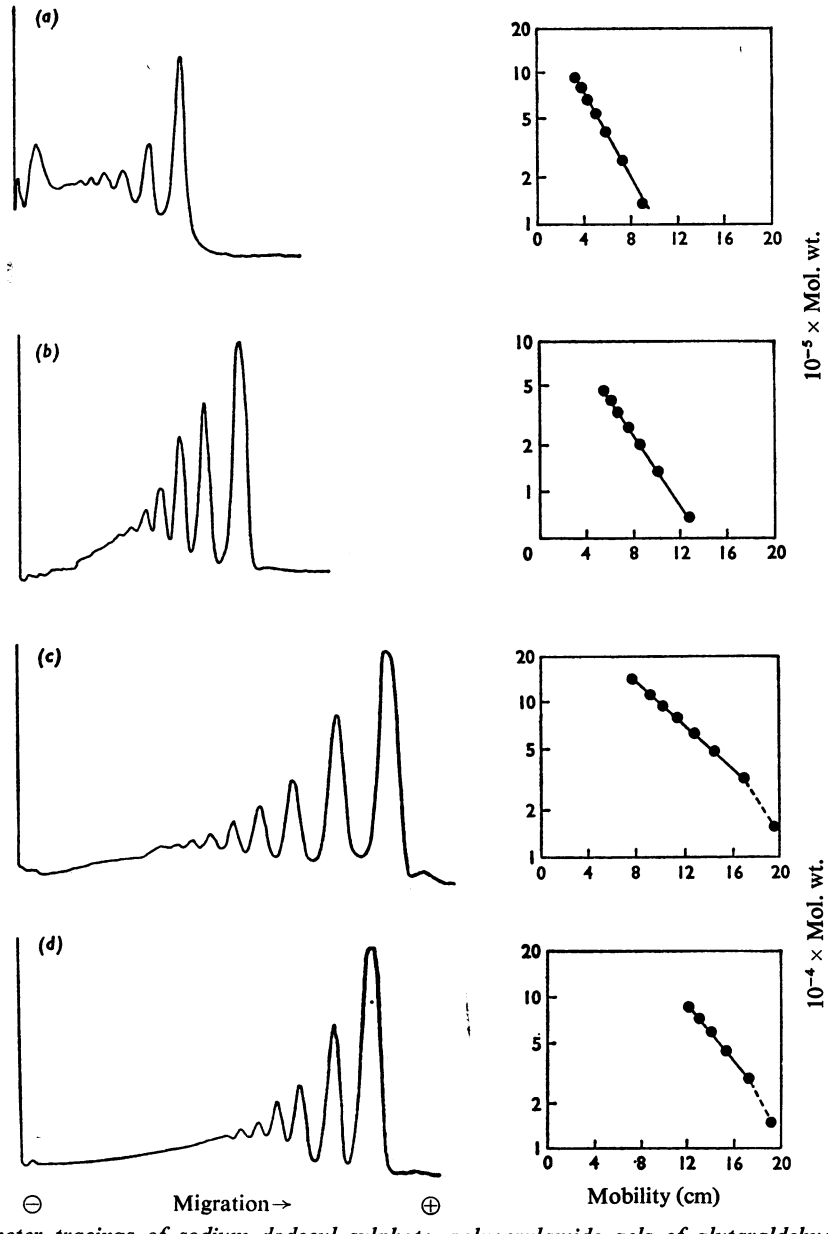


Fig. 1. *Densitometer tracings of sodium dodecyl sulphate–polyacrylamide gels of glutaraldehyde-polymerized proteins*

(*a*) Asparaginase (60μl, 125mg/ml) in 0.1M-sodium phosphate, pH7.5, was incubated with 1.0% glutaraldehyde (2μl) for 48h at 20°C. Product was diluted 11-fold before incubation with sodium dodecyl sulphate and a sample (30μg) was run at 8mA/gel for 165min. (*b*) Albumin (500μl, 125mg/ml) was treated with 5% glutaraldehyde (15μl) as in (*a*). Product was diluted 11-fold and a sample (30μg) was run at 8mA/gel for 135min. (*c*) Haemoglobin (50μl, 200mg/ml) was treated with 5% glutaraldehyde (2μl) as described in (*a*). Product was diluted 20-fold and a sample (30μg) was run at 5mA/gel for 210min. (*d*) Lysozyme (200μl, 50mg/ml) was treated with 5% glutaraldehyde (2μl) as described in (*a*). Product was diluted 3.5-fold and a sample (45μg) was run at 5mA/gel for 210min. Insets are plots of logarithm of molecular weight against mobility (cm) obtained from the tracings. Mobilities of various samples are not directly comparable as conditions varied slightly. Albumin (mol.wt. 67000), haemoglobin subunit (mol.wt. 16000), lysozyme (mol.wt. 14300; Weber & Osborn, 1969); asparaginase (mol.wt. 135000; Cammack *et al.*, 1972).

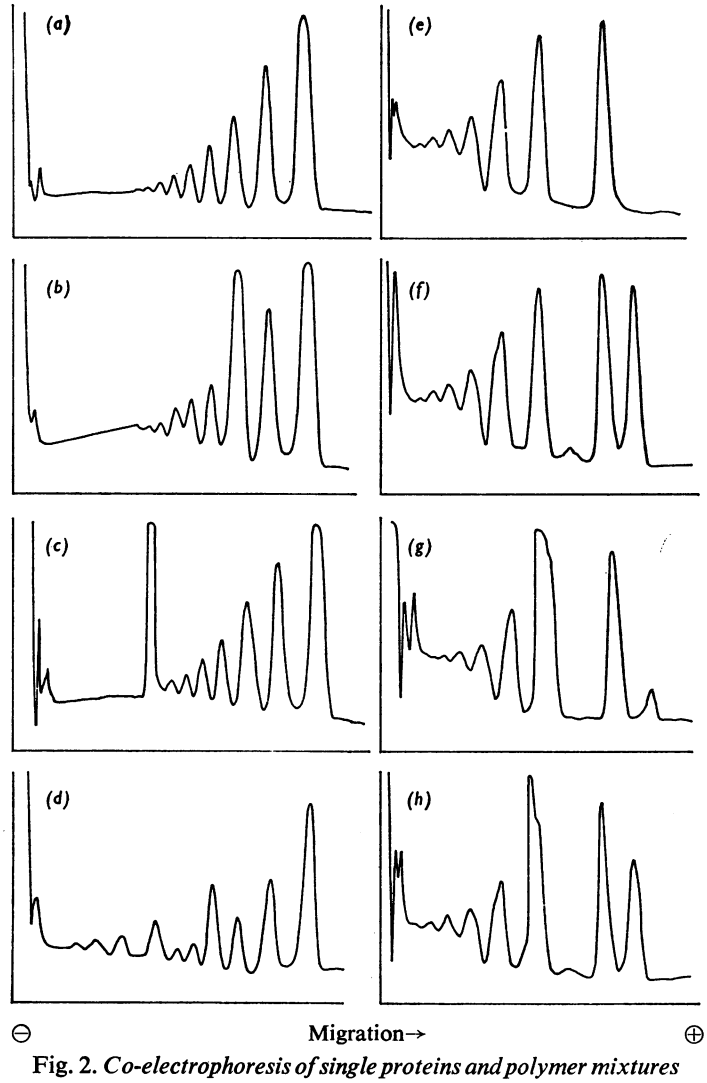


Fig. 2. *Co-electrophoresis of single proteins and polymer mixtures*

Samples were run at 5mA/gel for 3h (*a–d*) or 3.5h (*e–h*). (*a*) Haemoglobin polymers (45μg); molecular-weight distribution given in Fig. 1(*c*). (*b*) Haemoglobin polymers (35μg) plus ovalbumin (40μg) (mol.wt. 46000; Dunker & Rueckert, 1969). (*c*) Haemoglobin polymers (30μg) plus asparaginase (mol.wt. 135000). (*d*) Haemoglobin polymers (35μg) plus albumin polymers (25μg); albumin (5ml, 125mg/ml, mol.wt. 67000) was treated with 5% glutaraldehyde (165μl). (*e*) Albumin polymers (45μg) as in (*d*). (*f*) Albumin polymers (35μg) plus ovalbumin (30μg). (*g*) Albumin polymers (35μg) plus asparaginase (50μg). (*h*) Albumin polymers (30μg) plus asparaginase (25μg) and ovalbumin (25μg).

Co-electrophoresis of single proteins and polymer mixtures

The molecular weights of unknown proteins can be estimated conveniently by sodium dodecyl sulphate–polyacrylamide-gel electrophoresis in the presence of glutaraldehyde–protein oligomers. The internal oligomeric standards provide a calibration curve from which the unknown molecular weight may be interpolated. Quite simply therefore the range of available molecular-weight markers is extended enormously. A single gel may be adequate, but the use of several different oligomeric mixtures allows confirmation, and may facilitate location of the unknown protein as a discrete band between adjacent oligomers. The position of the unknown protein is easily revealed by its

asymmetric band relative to the oligomer pattern. Several examples of the technique are shown in Fig. 2 and the positions of the 'odd' proteins are apparent. The fastest moving peak in Fig. 2(*g*) corresponds to asparaginase monomer (mol.wt. 33500; Cammack *et al.*, 1972). In practice, it is convenient to improve separation of the oligomers by using longer gels and/or smaller amounts of protein. We have recently prepared sodium dodecyl sulphate–polyacrylamide-gel slabs rather than rods and have run them in a commercial 'Gradipore' electrophoresis cell. Samples similar to those in Fig. 2 gave good resolution and the fact that many samples could be run alongside each other simplified identification of protein bands and improved comparison of their mobilities.

Separation of glutaraldehyde–albumin oligomers on Sepharose 4B

For this 10% glutaraldehyde (165μl) was added in a single amount to a rapidly mixed solution of albumin (625mg) in 0.1M-sodium phosphate, pH7.5 (5ml), mixing was continued on a vortex mixer for 1min and the solution was then incubated at room temperature overnight. The product was dialysed against several changes of 0.01M-sodium phosphate, pH7.5, over 24h and samples (0.5ml) were fractionated on Sepharose 4B. An example of the protein pattern obtained is shown in Fig. 3, together with calibration markers of Blue Dextran and KI, and a control of untreated albumin. Blue Dextran is fractionated on Sepharose 4B but a definite peak corresponding to the void volume (about fraction 14) was observed. The bulk of the polymerized albumin also emerged in the void volume and would seem to be highly polymeric considering that proteins of about 2×10^7 daltons are excluded from Sepharose 4B (Pharmacia information booklet on Sepharose). This result therefore provides independent confirmation of the polymeric nature of the product. Certain of the fractions were examined by sodium dodecyl sulphate–polyacrylamide-gel electrophoresis. The high-molecular-weight polymers present in the early fractions are unable to penetrate the 5% gels and stain as sharp bands on top of the gels. Separation on the basis of size was achieved and fractions containing oligomers of particular molecular-weight distribution were obtained, the progression throughout the fractions from large polymers to monomer was clearly revealed. Fractionation of this type therefore provides the means to obtain polymers of a more narrow molecular-weight range (and by appropriate pooling to obtain them in comparable amounts with each other) that may be more suitable markers in particular circumstances.

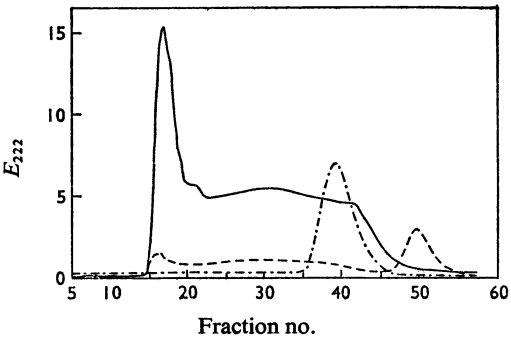


Fig. 3. *Separation of glutaraldehyde–albumin polymers on Sepharose 4B*

Polymer preparation is described in the text. The column of Sepharose (Pharmacia, 90cm×2.5cm diam.) was run at room temperature, at 29ml/h downwards under a constant head. A Gilford 2000 recording spectrophotometer, equipped with a flow cell (0.2cm path-width) was used to monitor the eluent at E_{222}. The three samples (0.5ml) were examined separately and eluted with 0.01M-potassium phosphate, pH7.5, containing 0.05M-KCl; fractions (45 drops; about 3ml) were collected. ——, Glutaraldehyde polymers (125mg/ml); – – – –, Blue Dextran (20mg/ml) plus KI (1mg/ml); –·–·–·, untreated albumin (25mg/ml).

Amino acid analyses of albumin polymers

Concentrated protein solutions were used in the experiments already described in the expectation that high-molecular-weight polymers with minimal amino acid substitution could be obtained, and evidence for this expectation was provided by determination of the relationship between the extent of amino acid modification and molecular size throughout the Sepharose fractions. These results are given in Table 1 and are compared with amino acid analyses of a control of untreated albumin, a sample of mixed polymers before Sepharose fractionation, and a sample of 'insoluble' albumin. This 'insoluble' albumin was prepared by adding 25% glutaraldehyde (100μl) with rapid mixing to a solution of albumin (1.0g) in 0.1M-sodium phosphate, pH7.4 (5.0ml). The solution rapidly turned yellow and after several min formed a solid gel. After incubation at room temperature (18h), ice–water (5ml) was added and the gel was macerated for 2min with a Sorvall Omnimixer. The resultant particles were washed six times with cold water (5ml) to remove soluble material and after centrifugation a sample of the sedimented gel particles was freeze-dried and hydrolysed for amino acid analysis. The effect of protein concentration on the nature of the product is well illustrated by this example in which a similar proportion (by weight) of

Table 1. *Modification of amino acids in albumin by glutaraldehyde*

Sample preparation is described in the text; protein samples were hydrolysed as described in the Materials and Methods section. Sample no. 1, untreated albumin; 2, polymer mixture (see Fig. 6); 3–8 are Sepharose fractions 15, 20, 24, 30, 34 and 40 respectively; 9, 'insoluble' albumin. The presence of oxidation products prevented accurate determination of methionine and cystine.

Amino acid	Sample no. ...	Relative amino acid compositions (Ala = 100)								
		1	2	3	4	5	6	7	8	9
Phe		67.7	67.0	63.6	58.0	59.7	64.5	66.0	68.0	67.0
Tyr		46.4	44.3	39.8	36.8	39.2	40.8	39.3	41.2	45.4
Leu		151.0	151.2	152.0	147.0	147.0	151.0	150.0	154.0	149.0
Ile		27.6	27.6	27.5	27.0	26.4	27.8	28.0	29.0	27.4
Val		69.0	69.5	68.4	68.0	68.0	67.0	65.5	61.2	68.8
Ala		100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Gly		43.2	43.0	44.2	44.5	43.6	41.0	42.2	44.8	44.2
Glu		184.0	184.0	186.0	179.0	185.0	185.0	182.0	186.0	187.0
Ser		63.8	64.0	66.0	68.0	68.4	64.5	62.9	65.5	65.6
Thr		72.4	71.7	72.6	73.8	68.4	72.3	71.4	71.8	71.6
Asp		122.2	122.4	121.0	121.0	118.0	122.0	121.0	120.6	117.0
Arg		55.3	56.6	52.0	46.3	46.0	53.2	45.8	41.2	53.6
His		44.1	43.5	42.7	38.7	35.4	41.2	43.9	39.2	44.0
Lys		141.0	123.0	123.0	109.0	105.0	118.0	121.0	120.5	121.0
Pro		33.8	33.9	34.0	34.0	32.2	33.2	32.8	33.5	32.8

glutaraldehyde was used, but 'insoluble' material resulted with albumin at 200mg/ml, whereas the soluble polymers examined on Sepharose were produced with albumin at 125mg/ml.

Reaction with glutaraldehyde causes mainly a decrease in lysine together with lesser losses of tyrosine, histidine and arginine (Table 1). The modified amino acids are acid-stable and are perhaps $\alpha\beta$-addition products rather than acid-labile Schiff-base complexes (Richards & Knowles, 1968). With the various Sepharose fractions (Table 1, samples 3–8) the extent of amino acid modification was similar in all samples irrespective of the degree of polymerization, indicating that extensive polymerization can occur with few intermolecular bridges. Similarly, the mixed polymers (Table 1, sample 2) closely resembles the individual Sepharose fractions, and the more extensively cross-linked 'insoluble' albumin is, in accord with the constant proportion of glutaraldehyde used, comparably modified. These analyses are of considerable significance for they endorse the idea that high protein concentrations and low amounts of glutaraldehyde can yield high-molecular-weight polymers of minimal substitution.

Discussion

The prime objective of this work was to devise a simple procedure to polymerize proteins with a minimal amount of amino acid modification. Previously, glutaraldehyde has been used mainly to insolubilize proteins, generally by using low (about 1mg/ml) protein concentrations and excess of reagent. Few systematic studies have been carried out and in particular little attention has been given to the influence of protein concentration on the nature of the products. Our results show the ease with which soluble polymers can be produced by using low concentrations of glutaraldehyde and high protein concentrations.

For use as molecular-weight markers these polymers should ideally be strict homologues. Evidence that extensive polymerization can be achieved with minimal amino acid substitution is implicit in the low amounts of glutaraldehyde employed, and is confirmed by the results (Table 1). That this polymerization may also be achieved with little conformational perturbation is indicated by further studies with albumin polymerized at 250mg/ml by using minimal amounts of glutaraldehyde. For example, on isoelectric focusing the products appeared as sharp bands of similar charge (H. K. Robinson, personal communication). The polymers were indistinguishable from native albumin when examined by optical rotary dispersion (D. I. Marlborough, personal communication), and only a small loss of antigenic determinants occurs (A. L. MacLennan, personal communication). In the ultracentrifuge, three distinct peaks were resolved with these polymers (K. A. Cammack, personal communication), and it is possible that such products may find application here also for calibration purposes.

Examination of various protein polymers on 'gradient-pore electrophoresis' was particularly noteworthy. This technique (Andersson *et al.*, 1972) uses gels of graded porosity into which proteins migrate under a potential gradient until they reach a 'pore-limit' related to their hydrodynamic volume. Although offering great potential as a means of estimating molecular weights, in our hands protein mobilities were too dependent on protein charge to allow accurate calibration or reliable results. However, with the polymers this objection was overcome and good calibration was achieved.

The adherence of the polymers to the inverse relationship between logarithm of molecular weight and mobility in sodium dodecyl sulphate–polyacrylamide-gel electrophoresis indicates that they may be useful as molecular-weight markers in this system. Carpenter & Harrington (1972) recently produced polymers by treating proteins with dimethyl suberimidate, and showed that they migrated in a manner consistent with a homologous series, which led the authors to suggest their potential use in determining molecular weights. In a related study, Griffith (1972) detailed circumstances under which polymers produced by glutaraldehyde treatment could migrate anomalously, and their use lead to inaccurate molecular-weight estimations. However, the materials he studied were in general rather extensively modified, and *a priori* one would expect extensive cross-linking to lead to anomalous migration. Awareness of such anomalies does not detract from the potential use of polymers with minimal cross-links.

The cheapness and intrinsic similarities of the type of polymeric proteins described offer advantages over a collection of relatively expensive proteins usually employed as molecular-weight markers. The use of polymers seems particularly valuable in the molecular-weight range above 75000, for which few markers are available. With haemoglobin discrete bands are seen in gels before staining, and the use of coloured markers may have particular uses, e.g. in columns used for gel-exclusion chromatography. Polymers labelled with azo, fluorescein or dansyl groups may also have advantages. We have not systematically explored the influence of parameters such as temperature and pH on product formation, although such studies may lead to improved products for use as molecular-weight markers. It is hoped that the emphasis of the present paper will encourage others to explore further experimental parameters and additional proteins that should provide an ideal collection of molecular-weight standards.

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